ACTA PATHOLOGICA
ET MICROBIOLOGICA
SCANDINAVICA
VOL 66

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

Vol 66 1966

Munksgaard Copenhagen 1966

Munksgaard
International Booksellers and Publishers Ltd
47 Prags Boulevard Copenhagen S Denmark



PRINTED IN DENMARK
VALD PEDERSENS BOGTRYKKERI
COPENHAGEN

The University Institute of Pathology (Head Professor Steen Olsen) and the 2nd University Clinic of Internal Medicine (Head Professor Knud Lundbæk), Kommunehospitalet Aarhus Denmark.

KIDNEY LESIONS IN RATS WITH SEVERE LONG-TERM ALLOXAN DIABETES

2 *Histochemical Studies Comparison with Human Diabetic Glomerular Lesions*

By

T STEEN OLSEN, H ØRSKOV and KNUD LUNDBÆK

Received 5 vii 65

When rats have been made diabetic by intraperitoneal or intravenous injection of alloxan, characteristic glomerular changes occur after 10 15 months of diabetes (*Ørskov, Nielsen, Steen Olsen, Rafaelsen & Lundbæk*, 1966)) In PAS stained preparations these changes manifest themselves as diffuse thickening of the capillary walls and mesangial regions, increasing in intensity to sausage like structures formed by glomerular capillaries filled with homogeneous or slightly fibrillar deposits with scattered lacunae containing residual endothelial cells, and in certain cases with lipid containing vacuoles In the early stages the changes look just like the diffuse glomerular lesions in human dia betes, while in more advanced stages they differ from it in lacking typical nodules and in not showing PAS-positive, hyaline thickening of the arteriolar walls which is an invariable finding in human dia betic nephropathy

In the present paper the histochemical characteristics of experi mental glomerulopathy will be compared with human diabetic ne phropathy

MATERIAL AND METHODS

The details of the experimental conditions were described in the above mentioned paper It may be recapitulated here that 49 rats were rendered diabetic by intra peritoneal or intravenous injection of alloxan some of them under kidney protec tion" (clamping the renal pedicle during and for 5 minutes after the injection) 36 non diabetic rats served as controls Some of the controls had clamping of the renal pedicle without injection of alloxan others were given insulin The induced glomerular changes were distributed in relation to the duration of diabetes as shown in Table 1

Aided by grants from the *Novo Foundation* the *F L Smith Jubilee Foundation* and the *Danish Science Foundation*

We wish to express our gratitude to Mrs *Ingeborg Filertsen* for skillful technical assistance

TABLE 1

Numbers of Animals with Glomerular Lesions

Grades of severity	Controls	Diabetic rats Duration of diabetes 5 mo	 10 mo	 15 mo	Total
+++	0	0	0	5	5
++	0	0	3	2	5
+	1	0	12	0	13
0	35	22	5	0	62
Total	36	22	20	7	

From *Orskov et al* (1966)

The histochemical studies were carried out on rats with grade ++ and +++ glomerular lesions (*vide infra*) Besides the same techniques were employed on one rat from each of the control groups and of the groups in which the kidney showed only mild diffuse capillary wall thickening in the glomeruli (grade +) For comparison the reactions were also done on human kidneys from 6 patients with long-term diabetes and characteristic diabetic glomerular changes noted in the light microscope

The stainings and reactions were performed according to methods described in current manuals of histochemistry (*Gomori* 1953 *Pearse* 1960 *Barka & Anderson* 1963, *Romeis* 1948 *Lison* 1960)

The digestion experiments were carried out by incubation of the deparaffinized sections for 60 min at 37° C in 0 05 M phosphate buffer, pH 8 9 containing 0 1 [illegible] for 3 hours at 37° C in 0 02 N HCl pH 1 6 [illegible] both instances, control sections were in- [illegible] and digested sections were then stained [illegible] upled tetrazonium

Histological Changes observed by Light Microscopy

A detailed description of the microscopic changes found in the various grades of nephropathy in the experimental groups has been published elsewhere (*Orskov et al* (1966), Fig 1, 2, 3) They may be summed up as follows

Grade + This lesion, the mildest observed in the series, is a diffuse thickening of the capillary walls in the glomeruli, especially in the *mesangial regions These changes are most distinct in* PAS stained preparations Although these changes were mild, they were unquestionable When the total series of preparations was examined without knowledge of the protocol numbers, they were easy to pick out

Grade ++ This means widespread, diffuse thickening of the capillary walls like grade + changes, but accompanied by more localized and thicker deposits in a few glomeruli, in the mesangial regions as well as peripherally in the lobuli

Grade +++ Apart from widespread, severe, diffuse thickening of the capillary walls, 10 60 per cent of all glomeruli show total occlusion of several capillaries in the PAS stained preparations This gives rise to vermiform or sausage-shaped structures, *lined by the original capillary walls*

Grade + + and + + + changes were not observed in the control groups Only one control rat had a grade + lesion

None of the experimental rats exhibited typical nodular changes of the kind seen in severe glomerulosclerosis in diabetic patients

RESULTS

The results are shown in Table 2

Routine Stainings General Reactions

The deposited material is eosinophilic and stains a yellowish brown with van Gieson van Gieson stained and especially trichrome Masson stained preparations show a characteristic difference between the colour of the moderate diffuse vascular wall changes (grades + and + +) on the one hand and the severe sausage like changes (+ + +) found only after a duration of diabetes of 15 months on the other With trichrome-Masson the former are blue and the latter a bright red Compared with human diabetic glomeruli thus the colour is more like the reddish blue exudative changes (in fibrin caps and arteriolar hya line) than the diffuse and nodular changes both of which stain a pure blue (Fig 4 6)

Unstained preparations examined by fluorescence microscopy in blue light and ultraviolet light showed only faint autofluorescence without a characteristic difference between experimental and human glomerular lesions

Polarization microscopy did not show birefringence except in frozen sections from the few glomeruli which contained lipids in which there were a few needle shaped birefringent crystals In human material too birefringent material is found only in connection with lipid ma terial No birefringent collagen fibrils were observed neither in the experimental lesions nor in human glomeruli

Reactions for Proteins

The deposits contain a moderate quantity of a protein component positive to Millon s reaction the DDD method for —SH groups and the coupled tetrazonium method There seems to be a greater content of protein in the severe experimental lesions (grade + + +) and in the human exudative ones than in milder experimental lesions (grade + +) and in human diffuse and nodular lesions (Fig 7 8) Corre spondingly Mallory s PTAH reaction for fibrin was highly positive

Digestion experiments showed that all the human diabetic lesions are *trypsin* resistant The experimental changes show resistance when followed by the non specific Masson trichrome staining but the severe lesions change colour staining red without digestion blue after tryp sin With the coupled tetrazonium reaction the results were rather varied indicating a moderate sensitivity to trypsin in the mild as well

1

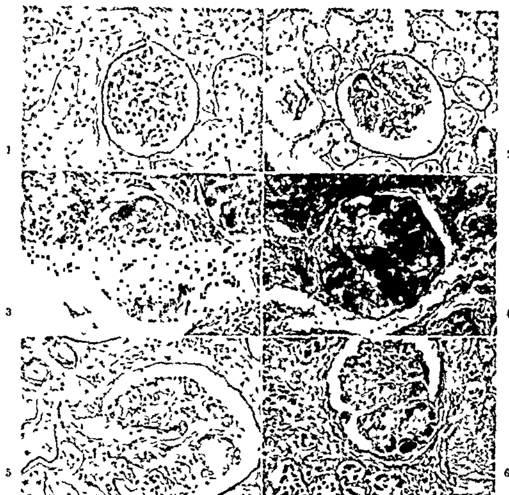
TABLE 2

Results of Staining and Histochemical Reactions.

Fo: formalin fixation, C fixation by perfusion with Carnoy's fluid, P: paraffin embedding, F: frozen section. 0–+++: semiquantitative estimate of the intensity of staining. Brackets indicate that only some of the structures are stained — var: variable staining intensity or colour or orthochromatic staining

	Fixation	P/F	Alloxan diab. rats.		Human diabetes			Remarks
			+ and ++	+++	dif.	nod.	exud.	
A Routine stainings general reactions								
Haematoxylin-eosin	Fo C	F P	+ red	+ red	+ red	+ red	++ red	
van Gieson-Hansen	Fo C	F P	++ brown	+++ orange	+ red	+ red	++ yellow	
Masson-trichrome	Fo C	F P	++ blue	+++ red	++ blue	++ blue	+++* red/blue	* var.
Unstained fluorescence, uv	Fo	F	+ blue	+ blue	+ blue	+ blue	++ blue	
Unstained fluorescence, blue	Fo	F	+ yellow	+ yellow	+ yellow	+ yellow	+++ yellow	
Unstained polarisation	Fo	F	0	(+)	0	0	(+)	only in lipids
B Reactions for special proteins or protein fractions								
Coupled Tetrazonium react	Fo C	P	+	++	+	+	++	
Trypsin digestion-Tetrazon	Fo	P	0–+	++*	+	+	++	* var.
Pepsin digestion-Tetrazon	Fo	P	0–+	+	0–+	0–+	+	
Trypsin digestion-Masson	Fo	P	+ blue	++ blue	++ blue	++ blue	+++* red/blue	* var.
Pepsin digestion-Masson	Fo	P	+ blue	++ blue	+ blue	+ blue	++ blue	
Millon's reaction	C	P	+	++	+	+	++	
DDD reaction for SH groups	C	P	+	+	+	+	+	
Mallory's PTAH	C	P	0	+++*	0	0	+++*	deep blue * var
Gallocyanin-chromalum	C	P	0	0	0	0	0	

carbohydrates								
Periodic acid Schiff (PAS)	Fo	I	++	+++	++	++	+++	++ = like normal basem. membr.
Periodic acid Schiff (PAS)	C	P	++	+++	++	++	+++	++ = like normal basem. membr.
PAS after acetylation	Io	I	0	0	0	0	0	
PAS after acetylation	C	P	0	0	0	0	0	
PAS after Diastase digestion	Fo	I	++	+++	++	++	+++	
PAS after Diastase digestion	C	P	++	+++	++	++	+++	
Best's Carmine	Io	P	0	0	0	0	0	
Alcian blue	Io	F	+	0	+	0	0	+ = like normal basem. membr.
Alcian blue	C	P	(+)	0	(+)	0	0	+ = like normal basem. membr.
Toluidine blue	Io	I	+ or	+ or	+ or	+ or	+ or	
Toluidine blue	C	P	+ or	+ or	+ or	+ or	+ or	
Astra blue	C	P	+	0	+	0	0	+ = like normal basem. membr.
Methyl violet	C	P	++ or	++ or	+ or	+ or	+++ or	
Congo red	C	P	0	0	0	0	0	
Congo red fluorescence uv	C	P	+ blue	+ blue	+ blue	+ blue	++ blue	like unstained fluorescence
Congo red polarisation	C	P	0	0	0	0	0	
Toluidine blue fluoresc. uv	C	I	+ blue	+ blue	+ blue	+ blue	++ blue	
Thioflavin fluorescence uv	C	F	+ blue	+ blue	+ blue	+ blue	+ blue	
D *Stainings for lipids*								
Sudan III	Io	F	0	(++)	0	(+)	(+++)	
Fettrot (CIBA)	Io	I	0	(++)	0	(+)	(+++)	
Copper phthalocyanin	Io	F	0	0	(+)	(++)	(+++)	
Phosphomolybdic acid (Landing)	Fo	F	0	0	0	0	(++)	
Phosphine 3 R	Fo	I	0	0	0	0	(++)	
Sudan Black B	Io	P	0	0	0	0	0	



Figs 1–6

Fig 1 Normal glomerulus from control group PAS haematoxylin
Fig 2 Glomerulus from rat with alloxan diabetes of 10 months duration Slight diffuse thickening of capillary wall PAS haematoxylin
Fig 3 Severe glomerular lesion from rat with alloxan diabetes of 15 months duration PAS haematoxylin
Fig 4 Same rat as Fig 3 Trichrome Masson
Fig 5 Glomerulus from *human* case of long term diabetes Typical nodular and arteriolar lesions PAS haematoxylin
Fig 6 Same case as Fig 5 Trichrome Masson Nodular lesions blue fibrinoid caps red

as in the severe experimental lesions After *pepsin* digestion there was a distinct decrease in the staining intensity, with Masson as well as with coupled tetrazonium, and this applied to all types of lesions in the patients as well as in the rats The red colour of severe or fibrinoid lesions was lost

The results of gallocyanin chromalum staining and Feulgen s reaction revealed that nucleic acid was not present in the deposits



Figs 7–12

Fig 7 Rat with severe glomerular lesion Coupled tetrazonium
Fig 8 *Human* case for comparison with Fig 7, coupled tetrazonium Fibrinoid caps and arteriolar hyalin are more intensely stained than nodular lesions
Fig 9 Rat,
Fig 10 *Hum*
Fig 11 Rat
Fig

Reactions for Carbohydrates

The deposited material in the capillary walls is highly PAS-positive (Fig 2, 3) This reaction was not reduced in strength following dia-stase digestion, but was totally blocked by acetylation It is somewhat stronger in the severe experimental and in the human exudative than in other types of lesions Staining with Alcian Blue, Toluidine Blue, (Fig 9, 10), and Astra Blue shows no content of *acid mucopolysacchar-*

ides apart from the sparse reaction normally seen in basement membranes. The PAS positive reaction was present following extraction of lipids and is not due to glycogen. These findings are identical in experimental and human lesions.

All reactions for *amyloid* were negative (Congo, Congo fluorescence, Congo polarization, Toluidine Blue fluorescence, Thioflavine fluorescence). This applied to rats as well as to patients.

The negative results of the reactions for acid muc polysaccharides and for amyloid in human diabetics apply to the present 6 cases. It must be added however that in rare cases we have seen positive reaction for acid mucopolysaccharides in human nodular and especially fibrinoid lesions. Metachromasia and staining with Astra Blue is always demonstrable in the intima of the larger arteries and in the interstitial tissue especially of the medulla—which affords a control that the reactions are successful. None of our experimental lesions was positive for acid mucopolysaccharides in the glomeruli but all were positive in the interstitial medullary tissue.—We have observed only once a human case giving a positive reaction to Congo red in nodules and arterioles. In this case there was no metachromasia with methyl violet.

Accordingly the PAS reaction in experimental as well as in human diabetic glomerular lesions must be ascribed to a carbohydrate component which is not glycogen, amyloid or acid mucopolysaccharides.

Stainings for Lipids

The severe experimental and human lesions occasionally show lipid content when stained with Sudan III (Fig. 11, 12) and Fettrot on frozen sections. The reactions differ widely from one glomerulus to another. The reaction for phospholipids and choline containing lipids is negative in an experimental material, while in a human material the former is occasionally positive. In severe experimental lesions the lipid content is localized apparently to the vacuoles mentioned above. In human diabetic lesions the lipid appears to be connected with the glycoprotein complex: it is present in largest quantities in the fibrinoid lesions. Investigation of human frozen sections in polarized light occasionally reveals maltese cross birefringence in the glomeruli, presumably due to cholesterol. A maltese cross birefringence was not observed in experimental glomerular lesions.

DISCUSSION AND CONCLUSIONS

Histochemical investigations into human diabetic glomerulopathy have been performed previously, int. al. by *Allen* 1941, *Randerath* 1953, *Muirhead, Montgomery & Booth* 1956. Most recently *Randerath, Diezel & Pfleiderer* 1959 have made a thorough study of the histochemical nature of the *nodular* changes and the *fibrinoid* lesions which include (1) droplet shaped or more flat thickenings on the inside of Bowman's membrane, (2) cap formations on glomerular capillaries and (3) ar

teriolar hyaline These authors established that the fibrinoid glomerular changes were histochemically identical They conclude that the nodular changes consist of high polymeric (only slightly acid) mucopolysaccharides with a low content of protein The fibrinoid changes were characterized by an ample content of proteins, carbohydrates, and lipid *Allen* (1941) demonstrated that nodular Kimmelstiel-Wilson changes were resistant to trypsin unlike the trypsin labile hyalinized arterioles *Koss* (1952), one of the first to study the nature of the fibrinoid lesion, found that it stained with all known fibrin stains (*Weigert, PTAH, van Gieson*), but did not have the structure of fibrin

Hatch, Watt, Kramer, Parrish & Howe 1961 did not find any signs of acid mucopolysaccharides, neither in diffuse nor in nodular lesions (negative Alcian blue, Hale), but ascribed the positive PAS reaction to ample quantities of neutral mucopolysaccharides

Most authors have found a negative amyloid reaction (*Allen* 1941 crystal violet, *Muirhead et al* 1956 Congo red, *Koss* 1952, methyl violet) This applies, in particular, to the nodular changes In fibrinoid lesions, however, *Muirhead et al* 1956 found a positive reaction, but only to Congo red staining of frozen sections

Thus, despite minor divergencies, it seems reasonable to conclude that previous investigations, especially those from recent years, as well as the present study show that *human diabetic glomerulopathy* is characterized by deposition of a substance having a high content of a carbohydrate which is not acid mucopolysaccharide and not amyloid In addition, diffuse and nodular lesions contain a fairly sparse protein component There may be varying, as a rule rather scanty, amounts of lipid, mainly in advanced diabetic nephropathy Capsular drops, capshaped deposits on the glomerular capillaries and arteriolar hyaline contain larger quantities of carbohydrate and protein, but are, in addition, characterized especially by a distinct reaction for fibrin and a high content of lipid

The light microscopic architecture of the kidney in *experimental alloxan diabetes* in rats has been studied by *Curtis, Robbins & Glickman* 1947, *Beveridge & Johnson* 1950, *Mann, Goddard & Adams* 1951, *Janes* (1959), and *Greenberg* 1962

Foglia, Mancini & Cardeza 1950 studied pancreatectomized diabetic rats *Beaser, Sak & Sommers* 1963 and *Beaser, Sak, Donaldson, Mc Laughlin & Sommers* 1964 have studied the kidneys of alloxan diabetic hamsters (*Mesocricetus auratus*) In addition there have been a few studies on experimental hormone provoked diabetes In the following we shall discuss only alloxan induced diabetes and diabetes provoked by pancreatectomy in which the pathogenetic conditions are considerably simpler than in experimental pituitary and cortisone induced diabetic metabolic anomalies

Only a few histochemical investigations into the glomerular changes in experimental diabetes have been published *Foglia et al* 1950 studied

the deposited PAS-positive material with thionine and toluidine blue as well as following hyaluronidase digestion They found no acid mucopolysaccharides The amyloid reaction to iodine was negative, and Sudan staining showed no lipids *Greenberg* (1962), on the other hand, has reported the presence of acid mucopolysaccharides represented by a positive Abul-Haj reaction

Histochemical studies will rarely give an exact chemical definition of a morphological structure The object of our experiment was to (1) perform a rough chemical classification of the components of the glomerular lesions and (2) to compare the results of the reactions found in experimental and in human diabetic glomerulopathy

With respect to the *nature* of the material deposited in the glomerular capillaries in alloxan-induced diabetes in rats, it may be said that it is a protein-carbohydrate complex whose carbohydrate component is neither represented by glycogen, amyloid, nor by acid mucopolysaccharides In severe lesions (grade + + +) there may be lipid deposits, *mainly in the form of lipid droplets in vacuoles The severe* lesions stain partially as fibrin

Digestion experiments with the purpose of characterizing the protein component gave somewhat varying results, but in general there was more resistance to trypsin than to pepsin digestion, and this applies to the mild as well as to the severe lesions Digestion with trypsin and pepsin alters the colour of the severe reactions from red to blue with trichrome-Masson staining

Comparison of the experimental lesion and the human diabetic glomerulopathy permits the following conclusions The histochemical nature of the diffuse mild and moderate grade lesions (+ and + +) in the experimental rats and of the diffuse and nodular lesions in human glomerular changes showed striking similarities when tested by the reactions used The more severe lesions (grade + + +) in experimental rats after 15 months of diabetes differ from diffuse and nodular lesions in diabetic patients in (1) a different result of certain non-specific staining reactions (van Gieson, trichrome Masson) (2) a somewhat more pronounced protein reaction, (3) a highly positive reaction for fibrin, and (4) occasional deposits of neutral fat However these characteristics apply, to a great extent, to the "fibrinoid" human lesions (capsular drops, fibrinoid caps, and arteriolar hyaline)

Accordingly, the following resemblances between human diabetic glomerulopathy and the experimental lesions were observed

(1) Structural and histochemical similarity between diffuse thickening of the capillary walls in man and experimental rats (grades + and + +)
(2) Histochemical similarity between human fibrinoid lesions and severe (grade + + +) lesions in experimental rats

The following differences were observed

(1) The diabetic glomerulopathy in man is (at least in advanced cases) always accompanied by hyaline-fibrinoid arteriolar wall thickening, most typical in the vasa afferentia and efferentia The rats did not exhibit a corresponding hyalinization of the arteriolar wall

(2) Typical nodules were not observed in the experimental rats (compare Figs 3 and 5)

No one has so far been able to induce diabetic lesions in animals, identical with the appearances in man Indeed, it is doubtful whether this can be expected at all in view of the species difference It must be reasonable to interpret the glomerulopathy in the alloxan diabetic rat tentatively as being analogous to the human glomerulopathy, because it is definitely induced by the diabetic metabolic anomaly and because —although the identity is not complete—it does exhibit striking similarities to human diabetic glomerular changes

SUMMARY

Glomerular lesions were observed in the kidneys of alloxan diabetic rats after 10 15 months' disease These lesions are histochemically characterized and compared with the lesions in the glomeruli in 6 human cases of long-term diabetes The experimental glomerulopathy consists of deposits in the glomerular capillaries of a glycoprotein complex which does not contain glycogen, amyloid or acid mucopolysaccharides A few of the most severe lesions contained lipid Similarities and differences between experimental and human lesions are described

REFERENCES

Allen A C So-called intercapillary glomerulosclerosis—a lesion associated with diabetes mellitus morphogenesis and significance Arch Path 32 33 51, 1941

Barka T & Anderson P J Histochemistry Theory practice and bibliography Harper & Row New York 1963

Beaser S B Sak M F & Sommers S C Influence of insulin therapy and pyelonephritis upon diabetic glomerulosclerosis in hamsters Metabolism 12 704 709 1963

Beaser S B Sak M F Donaldson G W McLaughlin R J & Sommers S C Alloxan diabetes in the golden hamster Mesocricetus auratus II Diabetes 13 49 53 1964

Beveridge J M R & Johnson S E Studies on diabetic rats The production of cardiovascular and renal disease in diabetic rats Brit J Exper Path 31 285–293 1950

Curtis G W Robbins S L & Glickman I Studies on glycogen nephrosis in alloxan treated diabetic rats J Exper Med 85 373 379 1947

Foglia V G Mancini R F & Carde a A F Glomerular lesions in the diabetic rat Arch Path 50 75–83 1950

Gomori G Microscopic histochemistry Principles and practice University of Chicago Press Chicago 1953

Greenberg S R Glomerular changes in chronic alloxan diabetes Arch Path 73 263 273 1962

the deposited PAS positive material with thionine and toluidine blue as well as following hyaluronidase digestion They found no acid mucopolysaccharides The amyloid reaction to iodine was negative, and Sudan staining showed no lipids *Greenberg* (1962), on the other hand, has reported the presence of acid mucopolysaccharides represented by a positive Abul-Haj reaction

Histochemical studies will rarely give an exact chemical definition of a morphological structure The object of our experiment was to (1) perform a rough chemical classification of the components of the glomerular lesions and (2) to compare the results of the reactions found in experimental and in human diabetic glomerulopathy

With respect to the *nature* of the material deposited in the glomerular capillaries in alloxan-induced diabetes in rats, it may be said that it is a protein carbohydrate complex whose carbohydrate component is neither represented by glycogen, amyloid, nor by acid mucopolysaccharides In severe lesions (grade + + +) there may be lipid deposits, mainly in the form of lipid droplets in vacuoles The severe lesions stain partially as fibrin

Digestion experiments with the purpose of characterizing the protein component gave somewhat varying results, but in general there was more resistance to trypsin than to pepsin digestion, and this applies to the mild as well as to the severe lesions Digestion with trypsin and pepsin alters the colour of the severe reactions from red to blue with trichrome-Masson staining

Comparison of the experimental lesion and the human diabetic glomerulopathy permits the following conclusions The histochemical nature of the diffuse mild and moderate grade lesions (+ and + +) in the experimental rats and of the diffuse and nodular lesions in human glomerular changes showed striking similarities when tested by the reactions used The more severe lesions (grade + + +) in experimental rats after 15 months of diabetes differ from diffuse and nodular lesions in diabetic patients in (1) a different result of certain non-specific staining reactions (van Gieson trichrome Masson) (2) a somewhat more pronounced protein reaction, (3) a highly positive reaction for fibrin and (4) occasional deposits of neutral fat However, these characteristics apply, to a great extent, to the "fibrinoid' human lesions (capsular drops, fibrinoid caps, and arteriolar hyaline)

Accordingly the following resemblances between human diabetic glomerulopathy and the experimental lesions were observed

(1) Structural and histochemical similarity between diffuse thickening of the capillary walls in man and experimental rats (grades + and + +)
(2) Histochemical similarity between human fibrinoid lesions and severe (grade + + +) lesions in experimental rats

The following differences were observed

Acta path. et microbiol. scandinav. 66: 13–30, 1966

The University Institute of Hygiene, Copenhagen (Chief: Poul Bonnevie, M.D.)

THE OCCURRENCE OF *NOSEMA CUNICULI* (*ENCEPHALITOZOON CUNICULI*) IN THE CELLS OF TRANSPLANTABLE, MALIGNANT ASCITES TUMOURS AND ITS EFFECT UPON TUMOUR AND HOST

By

MICHAEL PETRI

Received 1.ii.65

Two well known transplantable ascites tumours have for a number of years been used in experimental work at this institute: the Yoshida rat sarcoma and the Ehrlich mouse carcinoma (*Lund* 1955, *Petri* & *Lund* 1958). When almost three years ago the Yoshida sarcoma displayed obvious deviations from its habitual performance these were found correlated to the presence in the malignant cells of a parasite, presumably of the sporozoan order microsporidia (*Petri* 1965). Until lately these protozoa were believed not to occur in higher animals having been found mostly in arthropods and fishes, some having been demonstrated in still lower animals. The parasitized Yoshida sarcoma has till the present day (October 1964) been maintained in rats by serial transfers.

Evidence is presented below that the parasite found in the malignant cells is identical with *Encephalitozoon cuniculi Levaditi et al.* 1923 which due to the recent findings of *Weiser* (1964) and of *Lainson et al.* (1964), can be classified with certainty within the microsporidia, its proper name being *Nosema cuniculi* (*Levaditi, Nicolau & Schoen* 1923, *Weiser* 1964).

Some relationships will be described which arise when the infected sarcoma is introduced into a host animal, viz. that between the parasite and the tumour, that between the parasite and the host animal and that between the tumour and the host animal.

MATERIALS AND METHODS

Animals

A. Grown up male and female albino rats of the State Vitamin Laboratory strain kept on a standard diet. This strain dates back to 1919 (*Andreasen* 1943).
B. Grown up male and female albino mice, C-strain, kept on the same diet.

This work was supported by grants from the *Danish Anti Cancer League* and from the *Nordisk Insulin*...

Tumours

A Ehrlich mouse carcinoma kept by Lund at this institute for more than 20 years Intraperitoneal transfers with 0 1 ml undiluted ascites fluid Survival time 12 14 days

B Yoshida rat ascites sarcoma Intraperitoneal transfers with 0.3 ml subcutaneous inoculations with 0 5 ml undiluted ascites fluid Transplantation is usually done on the 5th day Survival time with the ascitic tumour 5–6 days with a subcutaneous tumour about 25 days Having observed the parasitic infection of this tumour an uninfected sample obtained from Professor *H Druckrey* Germany has been kept in serial transfers since 1961 No infection of these tumour cells has occurred till the present date (October 1964) in over 200 transfers

C The parasitic infection was discovered in a tumour which had been received from Germany[1] a few months previously Intraperitoneal transfers usually with 0.5 ml undiluted ascites fluid after about one week.

In vitro Cultivation

Before the definite nature of the parasite was established a number of attempts were made to cultivate the observed parasite in solid and fluid media The ascites was removed under aseptic conditions The following media were used meat extract agar Sabouraud agar blood agar ascites agar calf brain—beef heart—peptone agar (*Jepsen* 1960) Anaerobic cultivation was done using the last named medium in the B T L. anaerobic jar

Attempts to establish the parasitized sarcoma with the cultures obtained were done by intraperitoneal injection of 1 0 ml of a 24 hours pure culture in a corresponding but fluid medium at various intervals after intraperitoneal inoculation of the uninfected sarcoma.

Observation on Living Cells

A drop of ascites fluid was placed on a slide and gently spread out in a thin layer under a cover slip and then observed in the phase-contrast microscope The distance between the two glass surfaces is large enough to permit the cells to float around in the fluid

For studying polar *filament extrusion* under the influence of various chemicals a small drop of ascites fluid is spread out under a coverslip choosing a size which does not allow the edges of the drop to reach the edges of the coverslip The desired solution is then added at the edge of the coverslip

Staining Methods

The best structural differentiation of the parasite is obtained in a Giemsa stained *dry smear* of the ascites fluid fixed in absolute methanol Prolonged staining with dilute stain gives slightly better results

Wet smears fixed at 60° in Schaudinn s fluid and stained with Giemsa or with Heidenhain s hematoxylin in my hands have been inferior to Giemsa stained smears

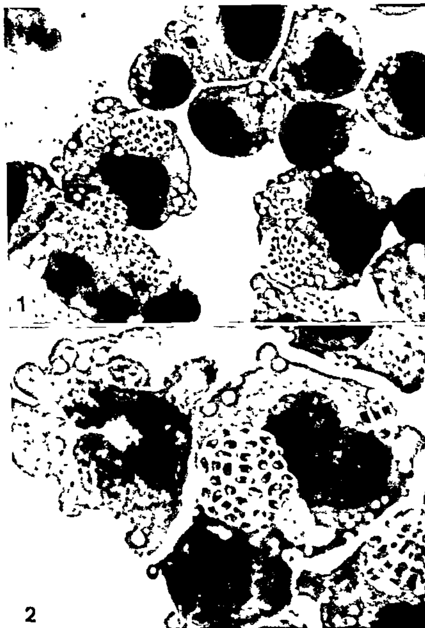
Tissues have been fixed in Zenker s fluid or Zenker acetic and stained by Wolbach s Giemsa-colophonium method (*Wolbach* 1919) or according to Levine (*Levine* 1961) The former method has given the best results though the difference is but small

RESULTS

In vitro Cultivation from the Ascitic Fluid

Within 72 hours only few colonies developed on the media used *B anitratum* and *Sarcina* were regularly, though not constantly found Besides various species of bacteria belonging to the *Enterobacteriaceae* were encountered now and then in small numbers Thus, very slight bacterial contamination was regularly found

[1] Trough the kindness of Professor Dr *Norbert Brock* Similar changes in the sarcoma had not been found at his laboratory (*Brock* personal communication)



Figs 1-2 Giemsa dry smears

Fig 1 Sarcoma cells with large parasitic vacuoles Two stages are seen in the two largest cells (1250 ×)

Fig 2 Closer view of spores The azurophil body of the parasite is well defined (1500 ×)

The ejection of the sporoplasm takes pl... cond and no impression has so far been ... m of extrusion. Ejection of the sporopla... hbouring cells has not been observed. Imm... the sporoplasm the filament curls up a ... wavy structure (Fig. 7)

...es used in this experiment no further d... m has been observed

... us Species of Rodents after Intraperitoneal ... e Infected Sarcoma

... twe... n the effect of the Yoshida sarcoma and the th... pathogenicity of the parasite was invest... a ... on of the infected sarcoma into sar... ... t... state had been produced by endocoer... d sarcoma (Lund 1957). In 4 rats this ... ed ascites sarcoma did not kill the animals ... action

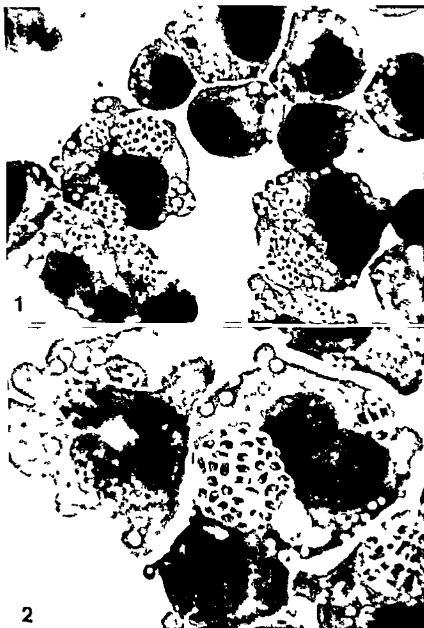
... ted rat sarcoma into other species of anima... survive obviously due to species differences ... be ascribed primarily to the parasite ... doses of 0.1–0.3 ml kill the animals in 6... ... ed 2 of 4 mice and 0.004 ml had no lethal ... lethal doses some mice after two to f... ... nts of ascitic fluid with moderate numbers ... ytes, a few of which contain *Nosema* spores ... er intraperitoneal injection of 2 ml

M...

Prelim... ary ... Tissues have been studied mainly during the acute stage of infection in rats just before or just after death from the parasitized sarc... or in mice inoculated with lethal doses of paras...s.

Histological stu... s of later stages are at present being done but have not yet permitted evaluation of the distribution of the parasite in various tissues, especially in the brain. The distinction between the first and later stages of infection may be important, as noted in studies of *Encephalitozoon* in mice by *Levaditi et al* (1924).

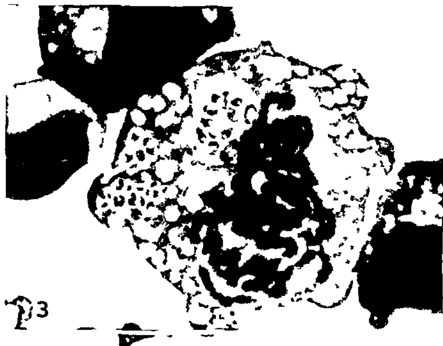
In the *rat* hepatosplenomegaly and ascites, usually 10–20 ml, are constant findings. The peritoneum is red and edematous, the parietal peritoneum is covered by heavy, fleshy deposits of fibrin. This violent production of fibrin can probably be attributed to the action of the parasite, as only very small amounts of fibrin can be found in rats at the same interval after inoculation of the uninfected ascites sarcoma. The liver may show different inflammatory changes, mostly patchy necro-



F gs 1 2 C emsa dry smears

F g 1 Sarcoma cells w th large para t c vacuoles Tv o stages are seen n the two largest cells (1250 ×)

F g 2 Cl ser vie v of sp res The azurophil l od of the parasite is v ell defined 1500 ×)





Figs 3–4 Giemsa dry smears
Prometaphase and telophase in parasitized sarcoma cells (2500 × and 1500 ×)

Morphology of the Parasite in Stained Preparations

Because of its small size only a few structural details of the parasite can be seen in the light microscope Nevertheless several stages of the parasite can usually be observed in dry smears of the infected sarcoma





Figs 5–6

Fig 5 Giemsa dry smears Spores in both nucleus and cytoplasm In the upper part of the cell a parasitic vacuole obviously is being formed (1500 ×)

Fig 6 The upper cell contains at least four rather indistinct stages of the parasite though partly inside a vacuole (1500 ×)

sporidia by *West* (1960) The ejection of the sporoplasm takes place within a fraction of one second and no impression has so far been obtained about the mechanism of extrusion Ejection of the sporoplasm into the same cell or neighbouring cells has not been observed Immediately after ejection of the sporoplasm the filament curls up and hence is seen as a more wavy structure (Fig 7)

Under the circumstances used in this experiment no further development of the sporoplasm has been observed

Pathogenicity in Various Species of Rodents after Intraperitoneal Injection of the Infected Sarcoma

To distinguish between the effect of the Yoshida sarcoma and that of the parasite in *rats* the pathogenicity of the parasite was investigated by intraperitoneal injection of the infected sarcoma into sarcoma-resistant rats The resistant state had been produced by endocoecal injection of the uninfected sarcoma (*Lund* 1957) In 4 rats thus vaccinated 2 ml of the infected ascites sarcoma did not kill the animals nor produce any visible reaction

By injection of the infected rat sarcoma into other species of animals the sarcoma cells do not survive obviously due to species differences, and any lethal effect must be ascribed primarily to the parasite

In *mice* intraperitoneal doses of 0 1–0 3 ml kill the animals in 6–10 days, whereas 0 02 ml killed 2 of 4 mice and 0 004 ml had no lethal effect in 8 mice After non-lethal doses some mice after two to four weeks produce large amounts of ascitic fluid with moderate numbers of lymfocytes and monocytes, a few of which contain *Nosema* spores

4 *guinea-pigs* survived after intraperitoneal injection of 2 ml

Morbid Anatomy

Preliminary studies Tissues have been studied mainly during the acute stage of infection, in rats just before or just after death from the parasitized sarcoma or in mice inoculated with lethal doses of parasites

Histological studies of later stages are at present being done, but have not yet permitted evaluation of the distribution of the parasite in various tissues, especially in the brain The distinction between the first and later stages of infection may be important, as noted in studies of "Encephalitozoon" in mice by *Levaditi et al* (1924)

In the *rat* hepatosplenomegaly and ascites, usually 10 20 ml are constant findings The peritoneum is red and edematous, the parietal peritoneum is covered by heavy, fleshy deposits of fibrin This violent production of fibrin can probably be attributed to the action of the parasite, as only very small amounts of fibrin can be found in rats at the same interval after inoculation of the uninfected ascites sarcoma The liver may show different inflammatory changes, mostly patchy necro



Fig 8

Rat wh ch has survi ed intraper toneal noculation of the paras tized sarcoma 4 months previ ously The anter or abdom nal wall has been opened and the organs d ssected apart to a certain extent The f brous t ssue can be seen as bands and sheets between the ntest nes At a few places bands have been left to show the adherence to the abdom nal wall Wh t sh masses are accumulated in the fibrous sheet cover ng the enlarged l ver The enlarged spleen in also shown

ses and a few liver cells contain groups of parasites The fatty tissue in the mesentery and the omentum is found invaded by sarcoma cells and thus parasites are numerous at these sites Usually the pleura con tains a small exsudate with sarcoma cells and inflammatory cells Macrophages and sarcoma cells may be invaded by parasites In the lungs scattered accumulations of lymphocytes and neutrophils are present at this time and in some cases metastatic sarcoma cells Eosinophilia occurs predominantly in the spleen

In *mice* inoculated with lethal doses of parasites similar changes in the liver spleen and lungs are found after death but the direct cause *of death or the spread of the parasite in the tissues has not yet been* found In the days following inoculation the parasite seems to multiply At about the same time usually after about six days sarcoma cells and almost all parasites disappear from the peritoneum After this the mouse dies

In rats which survive intraperitoneal inoculation of the infected sar coma the abdominal cavity is almost completely obliterated by fibrous agglutination (Fig 8) In this fibrous tissue yellow nodules of varying sizes are scattered mainly between the abdominal organs and the an terior abdominal wall Histologically these nodules consist of caseous

sporidia by *West* (1960). The ejection of the sporoplasm takes place within a fraction of one second and no impression has so far been obtained about the mechanism of extrusion. Ejection of the sporoplasm into the same cell or neighbouring cells has not been observed. Immediately after ejection of the sporoplasm the filament curls up and hence is seen as a more wavy structure (Fig. 7).

Under the circumstances used in this experiment no further development of the sporoplasm has been observed

Pathogenicity in Various Species of Rodents after Intraperitoneal Injection of the Infected Sarcoma

To distinguish between the effect of the Yoshida sarcoma and that of the parasite in *rats* the pathogenicity of the parasite was investigated by intraperitoneal injection of the infected sarcoma into sarcoma-resistant rats The resistant state had been produced by endocoecal injection of the uninfected sarcoma (*Lund* 1957). In 4 rats thus vaccinated 2 ml of the infected ascites sarcoma did not kill the animals nor produce any visible reaction.

By injection of the infected rat sarcoma into other species of animals the sarcoma cells do not survive obviously due to species differences, and any lethal effect must be ascribed primarily to the parasite.

In *mice* intraperitoneal doses of 0 1–0.3 ml kill the animals in 6–10 days, whereas 0 02 ml killed 2 of 4 mice and 0 004 ml had no lethal effect in 8 mice After non-lethal doses some mice after two to four weeks produce large amounts of ascitic fluid with moderate numbers of lymfocytes and monocytes, a few of which contain *Nosema* spores

4 *guinea-pigs* survived after intraperitoneal injection of 2 ml

Morbid Anatomy

Preliminary studies Tissues have been studied mainly during the acute stage of infection, in rats just before or just after death from the parasitized sarcoma or in mice inoculated with lethal doses of parasites

Histological studies of later stages are at present being done, but have not yet permitted evaluation of the distribution of the parasite in various tissues, especially in the brain The distinction between the first and later stages of infection may be important, as noted in studies of "Encephalitozoon" in mice by *Levaditi et al* (1924)

In the *rat* hepatosplenomegaly and ascites, usually 10 20 ml are constant findings The peritoneum is red and edematous, the parietal peritoneum is covered by heavy, fleshy deposits of fibrin. This violent production of fibrin can probably be attributed to the action of the parasite, as only very small amounts of fibrin can be found in rats at the same interval after inoculation of the uninfected ascites sarcoma The liver may show different inflammatory changes, mostly patchy necro-



Fig 8

Rat which has survived intraperitoneal inoculation of the parasitized sarcoma 4 months previously The anterior abdominal wall has been opened and the organs dissected apart to a certain extent The fibrous tissue can be seen as bands and sheets between the intestines At a few places bands have been left to show the adherence to the abdominal wall Whitish masses are accumulated in the fibrous sheet covering the enlarged liver The enlarged spleen in also shown

ses and a few liver cells contain groups of parasites The fatty tissue in the mesentery and the omentum is found invaded by sarcoma cells and thus parasites are numerous at these sites Usually the pleura contains a small exsudate with sarcoma cells and inflammatory cells Macrophages and sarcoma cells may be invaded by parasites In the lungs scattered accumulations of lymphocytes and neutrophils are present at this time and in some cases metastatic sarcoma cells. Eosinophilia occurs, predominantly in the spleen

In *mice* inoculated with lethal doses of parasites similar changes in the liver, spleen and lungs are found after death but the direct cause of death or the spread of the parasite in the tissues has not yet been found In the days following inoculation the parasite seems to multiply At about the same time, usually after about six days, sarcoma cells and almost all parasites disappear from the peritoneum After this the mouse dies

In rats which survive intraperitoneal inoculation of the infected sarcoma the abdominal cavity is almost completely obliterated by fibrous agglutination (Fig 8) In this fibrous tissue yellow nodules of varying sizes are scattered, mainly between the abdominal organs and the anterior abdominal wall Histologically these nodules consist of caseous

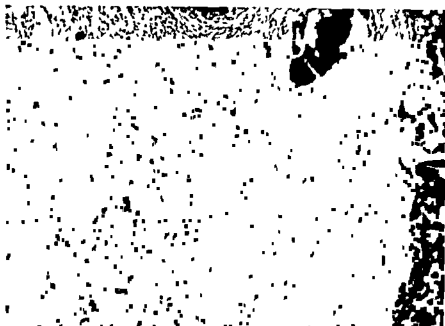


Fig 9

Tissue reaction around heavily calcified necrotic foci with histiocytes and giant cells H & I (160 ×)

necroses, often partly calcified, surrounded by a thin, fibrous membrane in which inflammatory infiltration is often slight, but sometimes with a rather pronounced admixture of lipid loaded macrophages and giant cells (Fig 9) In Giemsa-stained sections no parasites have been found Thus, intraperitoneal injection is no longer possible in these animals

Histological examination of the spleen, liver and abdominal lymphnodes from four such animals showed the pulp of the lymph nodes to consist almost entirely of plasma cells, and considerable plasma cell infiltration was also found in the red pulp of the spleen A great number of macrophages in these organs contained a brown pigment which gave a positive Prussian blue reaction for iron This pigment is presumably hematogenous It has been described in lymph nodes as a reaction to stress (*Selye & Foglia* 1939)

Behaviour of the Parasitized Sarcoma

When the infection of the Yoshida sarcoma was discovered it was regarded as an unfortunate event which rendered the tumour useless for experiments Naturally, any experiment in which the presence of the parasite is not realized will yeald misleading results

It was soon observed, however, that the infected tumour could be carried on by intraperitoneal transfers with preservation of as well

tumour cells as parasite When introduced into a new host both had the power of multiplication Thus two essential capacities of the sar coma were maintained, transplantability and malignancy

During the first year transfers were made at varying intervals and no attention was paid to cell counts Still the infected tumour was suc cessfully propagated Thus, the tumour parasite association is remarkably stable

In a rat usually from 6–10 ml of ascitic fluid can be withdrawn on the 5th day after inoculation Usually with 0 5 ml a total number of about 10^8 cells is inoculated but even low numbers, as 4×10^6 cells may give a successful transfer If care is taken to inoculate approximately 10^8 cells at each transfer the cell counts in the ascites fluid will mostly be between 150 000 and 200 000 cells per mm³ on the 5th day and the percentage of parasitized cells usually 15 to 25 per cent, as judged by Giemsa stained smears However, lower cell counts to about 50 000–70 000 per mm³, appear in some rats

When the infected tumour is inoculated into the peritoneum of a healthy rat it happens in order of frequency that

a) the rat dies,
b) the tumour disappears and the rat survives, developing resistance towards the uninfected sarcoma, or
c) the rat as well as the tumour survive, both for prolonged periods

a) Of 131 rats inoculated successively from December 1963 till October 1964 111 died (82 per cent) The survival time varied from 6 to 26 days, 75 per cent within 9 13 days No record of the non parasitized normal Yoshida sarcoma was kept during this period, but mortality was usually 98 99 per cent and survival time did not exceed 8 days

b) Initially all 20 surviving rats developed ascites with parasitized sarcoma cells Later, after disappearance of the ascites, these animals showed resistance towards the growth of sarcoma cells as none de veloped solid tumours after subcutaneous injections of the uninfected Yoshida sarcoma

Solid tumours never arise when the parasitized sarcoma is injected into the subcutaneous tissue of a healthy rat while the non parasitized sarcoma will nearly always yield a tumour at this site In six rats given three subcutaneous injections of the infected sarcoma no re sistance towards the non infected sarcoma arose, as subsequent intraperitoneal injection of the non infected sarcoma killed the animals in 6 to 8 days

c) In a few of the surviving animals the ascites does not disappear, but increases slowly until the abdomen is grossly distended The ascites contains a fair number of cells, around 50 000 per mm³ or less, in flammatory cells mostly lymphocytes and macrophages, as well as pale-staining sarcoma cells Less than 1 per cent of all cells contain various stages of the parasite This condition has been maintained

spontaneously for long periods in several rats in one rat for six months till the present date (October 1964) The animals appear to be in good health with their distended abdomens When ascitic fluid from these animals was injected into a healthy rat the latter produced an ascites sarcoma which killed it in 20 days while the donor rat continued in good health The percentage of parasitized cells was low but increased during subsequent passages

Although the disappearance of tumour tissue is an indirect sign of cytolysis this effect can be directly appreciated in a smear of the infected sarcoma Here a variable number of dead partly disintegrated cells can be observed the greatest numbers one to three days after inoculation into the peritoneum

DISCUSSION

The ejection from a polar vacuole of a sporoplasm attached to a polar filment classifies the parasite with the *Cnidosporidia*, a subclass of the protozoan class *Sporozoa* Due to the small size of its spores it belongs to the order *Microsporidia* According to *Kudo* (1954) Microsporidia with oval spores of this kind are classified as one family the *Nosematidae*

In his standard work on protozoology *Kudo* (1954) states that the Microsporidia are exclusively intracellular parasites of the lower vertebrates and invertebrates *i e* cold blooded animals or still lower organisms The most well known of the Microsporidia are some which cause wide spread and important diseases of insects *Nosema apis* and *Nosema bombi* cause epidemic diseases among bees They are known also in Denmark (*Hammer* 1946 *Skou et al* 1963) Another is *Nosema bombycis* causing the pebrine disease of silkworms Organisms invaded by Microsporidia are often severely affected (*Kudo* 1924)

It was peculiar therefore to find a parasite as d scribed in this paper in a warm blooded animal which could unquestionably be classified within the Microsporidia At the same time however other reports appeared which definitely proved the existence of microsporidian parasites in mice (*Nelson* 1962) and in the field mouse *Apodemus sylvaticus* (*Doby et al* 1963) Nelson could clearly demonstrate a polar filament and the attachment of a sporoplasm

Several small intracellular parasites similar to the one described in this paper are known from animals and man and which have all defied systemtic classification Foremost among these is *Toxoplasma gondii* Interest in the others have in the literature of recent years mainly been centered around their morphological similarity to this organism and thus their importance in the differential diagnosis of toxoplasmosis This group includes *Besnoitia* and M organism (*Frenkel* 1953 1956) and *Encephalitozoon* Among these *Encephalitozoon* is of the same size as the *Nosema* described in this paper it is Gram positive and its

morphology (*Wright & Craighead* 1922, *Perrin* 1943) very similar I have not had, unfortunately, other sections or smears with *Encephalitozoon* at my disposal

Encephalitozoon was first described in 1922 (*Wright & Craighead*) in rabbits as a cause of paralysis, found in the brain, kidney and urine It was studied and named *Encephalitozoon cuniculi* by *Levaditi et al* (1923) Infections of mice with ascites could be produced *Encephalitozoon* is commonly found in healthy laboratory mice and rats (*Perrin 1943*) One case of protozoan, human encephalitis attributed to Encephalitozoon has been reported from Japan (*Matsubayashi* 1959)

Of great interest in the present cases, however, was the fact that already *Levaditi et al* (1924) classified *Encephalitozoon* as a microsporidian based on its morphology in stained smears and sections Final proof was at that time lacking, as a polar filament was not demonstrated It was named "la microsporidiose de lapin"

The identification of *Encephalitozoon* as a microsporidian was recently suggested by *Nelson* (1962) from experiments with an ascites producing protozoan agent, resembling *Encephalitozoon* in white mice This parasite showed polar filaments and sporoplasms Final proof of this assumption, however, is now available in the work of *Lainson et al* (1964) These authods could demonstrate the extrusion of a filament from scanty parasites in the peritoneal cavity of mice and electronmicrographs of these spores showed the coiled filament The parasites were obtained by inoculation of brain suspensions from rabbits naturally infected with *Encephalitozoon*

These authors propose that the correct namt of *Encephalitozoon cuniculi, Levaditi et al* 1923, as found in various rodents and man is *Nosema cuniculi*

The Gram positive parasite described in the present work is identified similarly Although knowledge of its pathology, especially in the rabbit, is not yet available, the production of ascites in white mice and the proof of its classification as a *Nosema* at present substitute sufficient evidence Further, the fact that it produces ascites clinically only in a minority of the mice tested may be accounted for by species differences in different mouse strainsas noted by *Nelson* (1962) This ascites production is the best method available of demonstrating the presence of *Nosema cuniculi* in animal tissues, when done with pathogen free animals, as parasites can be found in smears of the mouse ascitic fluid Another method may be the infection of mouse ascites carcinoma cells when carcinoma cells and parasite containing material are introduced into the peritoneum of healthy mide

Weiser, however, proposed a new species, *Nosema muris* (*Weiser* 1965), based on a microsporidian parasite found by him in white mice He based this upon the organ distribution of his parasite which according to him differs from that of *Nosema cuniculi* and other microsporidia as reported in the literature, especially *Nosema cuniculi* (see

Weiser 1965) in not being found in the kidney but regularly in the myocardium Although *Nosema cuniculi* is known particularly as a kidney (and brain) parasite and has not been found in the myocardium in the rabbit a real picture of the organ distribution in the mouse is not furnished by the authors mentioned by Weiser As the detailed morphology of spores and vegetative stages of his parasite was not compared to that of these other Nosemas the difference in organ distribution may be a matter of susceptibility of the host during the observed stage of infection Although in the present work lesions of the kidneys have not been found in experimentally infected mice, while lesions of the myocardium seem to be more characteristic, this is considered inadequate evidence at the present time for establishing a new species

Nosema cuniculi is generally considered only weakly pathogenic towards healthy mice (*Perrin* 1943, *Frenkel* 1956, *Yost* 1958) The present investigation, however, shows that the pathogenicity of the parasite depends on dose This has been possible to demonstrate because the infected Yoshida rat sarcoma harbours great numbers of parasites, so the injection of 0 1 ml of this ascitic tumour into mice may introduce about 10^7 to 10^8 parasites As mentioned above this will kill the mouse in about 6–10 days, while 0 004 ml has no such effect Rats and guinea-pigs survived doses of 2 0 ml

It is known that *Nosema cuniculi* can be transmitted in mice by serial intraperitoneal transfers (*Perrin* 1943, *Lainson et al* 1964) The similarity of this mode of propagation to that of transplantable ascites tumours may in part account for the ease with which one tumour has been maintained by serial transfers though constantly parasitized by great numbers of *Nosema cuniculi*

It is therefore most desirable to know whether this may apply to other transplantable ascites tumours as well Investigations which are at present being done at this laboratory seem to support this possibility By intraperitoneal injection into mice of parasitized rat sarcoma cells and subsequent transplantation of the Ehrlich mouse ascites carcinoma the carcinoma cells were parasitized by *Nosema cuniculi*, although in small numbers, and the infected ascites carcinoma has so far been carried on by regular transfers for eight months Definite disturbance of the transplantability of this tumour has been noted but the results do not yet permit any conclusive evaluation

Considering the slight bacterial contamination sometimes found, heavier bacterial contamination might be thought to have caused the reduction of the number of cells which has been mentioned above This is reasonable since there is also a danger of puncturing the intestine in any experiment involving injection into the peritoneal cavity Although in vitro cultures have not been done at each transplantation of the parasitized sarcoma microscopic control has been made each time by phase contrast microscopy, and Giemsa stained dry smears of

nearly all tumour material used have been examined It is believed that any significant bacterial contamination would in this way have been brought to light It seems more reasonable to believe that the ascites producing property of the parasite which at least in mice is different in each animal may in some rats bring about a dilution of the tumour

Some features of the parasite tumour relationship as shown by the parasitized Yoshida rat ascites sarcoma are of special interest

It is evident that in spite of extensive multiplication of the para sites the tumour grows during this period *i e* the sarcoma cells divide and finally kills the animal As only a part of the sarcoma cells con tain visible parasites it is conceivable that the growth of the ascitic tumour is due to the multiplication of non infected cells Against this concept may speak the occurrence of parasitized cells in various even late stages of mitosis

Further as a large part of the malignant cells are not infected it is difficult to explain why subcutaneous inoculation is not followed by the growth of a solid tumour which happens when small amounts of the non infected sarcoma are inoculated into the subcutaneous tissue It is probable however that cells which are not visibly infected have at some earlier stage carried the parasite during its life cycle This assumption is partly based on the finding of large or small irregular empty vacuoles in the majority of non infected cells This particular morphology is almost never met with in smears of the uninfected sar coma They are interpreted as empty parasite vacuoles as their mor phology closely agrees with that of parasitic vacuoles The functional alteration in a cell which is produced by permanent or transient visit of *Nosema cuniculi* is not known but may account for the lowered growth potential at this site

It is apparent however that the parasite may have a cytolytic or in the present connection a cancerolytic effect This is born out by the fact that 20 per cent of rats inoculated with the infected ascites sar coma at first develop ascites with numerous sarcoma cells but as the ascites later disappears and the rat survives destruction of the sar coma cells seems to have taken place The appearance of resistance towards the normal uninfected sarcoma in such rats may be a kind of vaccination but it is at present unknown whether immunological mechanisms are active in this development

Spontaneous occurrence of rats resistant towards the Yoshida sar coma is well recognized as the tumour sometimes does not take by serial transfers and the inoculated cells are destroyed Although no record has been kept of this in the serial transfers of the normal un infected sarcoma at my laboratory I reckon that it happens in about 1 2 per cent of inoculated rats Persistance of the tumour in a surviv ing rat however has never been met with during several thousand transfers at this institute

It seems reasonable to believe, therefore, that the infection of the Yoshida tumour by *Nosema cuniculi* is in some way responsible for the prolonged survival of some rats with viable sarcoma cells, although the infection rate of such tumour cells is low. This peculiar phenomenon of adaptation between tumour and host is not solely due to a lowered malignancy of the sarcoma cells—whatever this may be—but at least partly to an altered reactivity at the host because when this tumour is transferred to a new host it reassumes its malignant behaviour and kills the host. When carried on by further transfers, the infection rate gradually increasing, this malignant pattern is kept.

Tumour tissue has been found—or reappears—not only in the ascitic form, but in one case as solid, intraabdominal tumours at least five months after inoculation of the infected sarcoma. The course of events in this period is unknown. In human malignancy the occurrence of late metastases, even years after removal of the primary tumour, have been ascribed to the activation of "dormant" cancer cells, but such cells so far seem to have escaped detection.

The portal of entry of *Nosema cuniculi* in the present case has not yet been found. *Encephalitozoon* is known to occur endemically in some stocks of animals (*Perrin* 1943) without signs of disease. In the period before or after infection was discovered, a short time after the Yoshida sarcoma was received from abroad, the parasite has, however, never been found in the two tumours kept at this institute. Both have been examined histologically at regular intervals. In lower animals the infection of the host tissues takes place via the gut, where the sporoplasms are released from spores and injected into the intestinal epithelium. Attempts to infect the ascitic rat sarcoma or mouse carcinoma by simultaneously feeding Nosema spores to the animal have as yet been unsuccessful in this respect.

That *Nosema cuniculi* will find a place in human pathology is not yet established with certainty. The human case described by *Matsubayashi*, however, was caused by a parasite which could not in dry smears and mouse passages be distinguished from *Encephalitozoon* though the microsporadian nature of this parasite was not considered. The structure and life cycle of the Microsporidia furnish important tools in the diagnosis of such human cases, if they exist, primarily because the demonstration of a polar filament classifies a parasite undoubtedly within this group. It may appear rewarding to keep this in mind when studying *e.g.* encephalitis of unknown etiology.

SUMMARY

1) For two and a half years a strain of the Yoshida rat ascites sarcoma has during serial transfers shown a heavy intracellular infection by a sporozoan parasite. The behaviour of this tumour closely mimics the behaviour of the normal uninfected Yoshida sarcoma.

2) The parasite appears to be identical with *Nosema cuniculi* (*Levaditi et al* 1923, *Weiser* 1964), formerly known as *Encephalitozoon cuniculi* (*Levaditi et al* 1923) It has not before been described in malignant tumours
3) Ejection of a sporoplasm attached to a polar filament from parasites in the sarcoma cells has been confirmed
4) Of various rodents tested *Nosema cuniculi* was found lethal towards mice, depending, however on the number of parasites inoculated Non lethal doses of parasites in some mice produce ascites in which parasitized inflammatory cells can be found
5) *Nosema cuniculi* is found in vacuoles in the malignant cells in great numbers Several stages can be recognised spore forms, however, always dominating
6) The parasitized ascites sarcoma is characterized by lower cell numbers *i e* excess fluid production, as compared to the non infected sarcoma In rats the ascites development is followed by a severe fibrinous peritonitis leading to complete obliteration of the peritoneal cavity in surviving animals
7) Of a large series of rats inoculated into the peritoneum with the parasitized sarcoma 20 per cent survived The infected ascites tumour, which appeared initially, disappeared These animals showed resistance towards subsequent inoculation of the non-infected sarcoma The survival rate with the non infected sarcoma is about 1 2 per cent in untreated animals
8) In a few rats survival was associated with the persistence of viable sarcoma cells for long periods up to six months This phenomenon has not been met with in over two thousand passages of the normal uninfected sarcoma in the same strain of rats
9) In surviving rats and mice the abdominal lymph nodes and the spleen showed large accumulations of plasma cells and a dark brown iron containing pigment was found in the reticular cells

REFERENCES

Andreasen E Studies on the Thymolymphatic System (Diss) Copenhagen 1943

Doby J M Alain Jeannes & Bernard Rault Thelohania apodemi n sp premiere microsporidie du genre Thelohania observee chez un mammifere C R Acad Sci (Par) 257 248 251 1963

Frenkel J K Infections with organisms resembling toxoplasma together with the

Jepsen Aa Diagnostisk Bakteriologi og Levnedsmiddelbakteriologi København 1960

Jirovec O Studien uber Microsporidien Mem Soc Zool Tchecoslov 4 5–79 1936

Kudo R A biologic and taxonomic study of the microsporidia Illinois Biol Monog 9 7 268 1924

Kudo R Protozoology 4th Ed Springfield Illinois 1954

Lainson R Garnham P C C Killick Kendrick R & Bird R G Nosematosis a microsporidial infection of rodents and other animals including man Brit Med J *II* 470 472 1964

Levaditi C Nicolau S & Schoen R L'étiologie de l encephalite C R Acad Sci (Par) *177* 985–988 1923

Levaditi C Nicolau S & Schoen R La microsporidiose du lapin ses relations avec la rage C R Acad Sci (Par) *178* 256 258 1924

Levine N D Protozoan Parasites of Domestic Animals and Man Minneapolis 1961

Lund H J C Isolation of a cancer specific antigen Danish Med Bull 2 73 82 1955

Lund H J C Vaccination of rats against the Yoshida ascites sarcoma with the formation of complement fixing antibody Brit J Cancer *11* 475–477 1957

Matsubayashi H Koike T Mikata I Takei H & Hagiwara S A case of Encephalitozoon like body infection in man Arch Path *67* 181 187, 1959

Nelson J B An intracellular parasite resembling a microsporidian associated with ascites in Swiss mice Proc Soc Exp Biol Med *109* 714 717 1962

Perrin T L Spontaneous and experimental Encephalitozoon infection in laboratory animals Arch Path *36* 559 567 1943

Petri M & Lund H J C Vaccination against the Yoshida rat sarcoma Acta path et microbiol scandinav *44* 106–111 1958

Petri M A cytolytic parasite in the cells of transplantable malignant tumours Nature *205* 302 1965

Selye H & Foglia C F On the formation of hemolymph nodes during the alarm reaction A J Anat *64* 133 1939

Skou J P Norgaard Holm St & Haas H Preliminary investigations on diseases in bumble bees (Bombus latr) R Vet Agr Coll Yearbook 27–41 1963

Weiser J On the taxonomic position of the genus *Encephalitozoon* Levaditi Nicolau & Schoen 1923 (Protozoa Microsporidia) Parasitology *54* 749 751 1964

Weiser J *Nosema muris* n sp a new microsporidian parasite of the white mouse (Mus musculus L) J Protozool *12* 78 83 1965

West A F The biology of a species of nosema (Sporozoa Microsporidia) parasitic in the flour beetle Tribolium confusum J Parasit *46* 747 754 1960

Wright J H & Craighead E M Infectious motor paralysis in young rabbits J Exp Med *36* 135–140 1922

Yost D H Encephalitozoon infection in laboratory animals J Nat Cancer Inst *20* 957 960 1958

Acta path et microbiol scandinav 66, 31-40, 1966

The Fibiger Laboratory, Lundtoftevej 5 Kgs Lyngby, Denmark
Head Jørgen Kieler Dr med

CULTIVATION OF MONOLAYER CELL CULTURES IN A CONTINUOUS PERFUSION SYSTEM

By

P BRIAND

Received 27 v 65

During the last two decades classical tissue culture methods have yielded a considerable amount of information about the nutritional requirements of animal cells in vitro One of the main purposes of these studies have been to develop well defined synthetic media which can support cell growth in vitro without the addition of the conventional, poorly defined biological media Much knowledge has been gained about the qualitative requirements of a number of cells and tissues, but it is difficult to assess the quantitative requirements of cells grown in conventional systems where the concentration of the nutrients is varying continously

Both the concentration at any given moment and the total supply of a nutritional factor during the whole experimental period may be of importance In order to establish the optimal concentration a system is required which rules out the total supply of the nutrient as a limiting factor

A continuous perfusion system may meet this requirement and many such systems have been described However, they are not equally suitable for cytological, biochemical and growth studies, and some of them require that the cells grow in suspension, which only few cell lines are able to do In the present experiments an attempt was made to grow a mouse ascites tumour in a system which combines the advantages of a perfusion system with the possibilities for cytological biochemical and growth studies offered by monolayer cultures grown on glass

MATERIAL AND METHODS

The cells studied were Landschutz hyperdiploid subline of Ehrlich mouse ascites carcinoma (ELD cells) These cells were originally adapted to *in vitro* conditions by *B Holmberg* at the Research Division of Radiumhemmet in Stockholm They have been propagated in this laboratory since 1962 in a medium composed of 10 per cent calf serum and 90 per cent synthetic medium In the present experiments calf serum was replaced by 20 per cent foetal bovine serum The synthetic medium was *Eagle* s

This work was supported by grants from the *Arvid Nilsson Foundation* and *King Christians Xth Foundation*

TABLE 1

Relationship between Medium Supply and Growth of ELD Cells in a Perfusion System

Medium supply	Control cultures*	2–4 ml/day	4–8 ml/day	8–12 ml/day	12 16 ml/day	16–24 ml/day	24–32 ml/day	> 32 ml/day
Number of experiments	24	16	29	18	26	10	14	14
Growth $\frac{C}{C_0}$ †	8 3 ± 2 0	16 7 ± 6 2	14 1 ± 3 4	23 7 ± 5 2	22 2 ± 4 2	28 3 ± 6 4	20 6 ± 4 4	30 0 ± 5 6

* Stationary cultures—no change of medium during experimental period
† Average rate of growth with 95 per cent confidence interval after 3 days of incubation C_0 = initial cell concentration   C = final cell concentration
ELD Ehrlich-Landschutz' hyperdiploid ascites tumour

TABLE 2

Relationship between Medium Supply and Growth of ELD Cells in a Perfusion System

Medium supply*	Control cultures†	3 10	10–20	20 30	30 40	40–60	> 60
Number of experiments	24	29	30	17	15	19	17
Growth $\frac{C}{C_0}$ §	8 3 ± 2 0	10 3 ± 2 8	19 3 ± 4 0	23 7 ± 4 6	23 5 ± 5 0	26 4 ± 4 2	30 9 ± 4 6

* Medium supply expressed as ml per day per 10^9 cells initially
† Stationary cultures which had no change of medium during experimental period
§ Average rate of growth with 95 per cent confidence interval after 3 days of incubation C_0 = initial cell concentration   C = final cell concentration
ELD see Table 1

TABLE 3

Relationship between Efferent Glucose Concentration and Growth of FID Cells in a Perfusion System

Glucose concentration	0 1 mM	1–2 mM	2 3 mM	3–4 mM	4 5 mM	5 6 mM	6 7 mM
Number of experiments	21	11	14	12	23	15	4
Growth $\frac{C}{C_0}$ *	12 6 ± 3 4	13 9 ± 6 0	24 9 ± 5 0	24 7 ± 8 8	26 8 ± 3 8	26 9 ± 5 4	30 5 ± 3 4

* Average rate of growth with 95 per cent confidence interval C_0 — initial cell concentration C — final cell concentration after 3 days of incubation
FID see Table 1

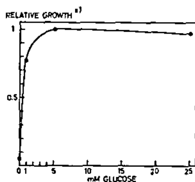


Fig 2

Relationship between afferent glucose concentration and growth of ELD cells in a perfusion system

*** Growth is relative to growth at a glucose concentration of 5 millimoles per litre**

a significant retardation of cell growth occurred when the glucose concentration of the efferent medium fell below 2 millimoles per litre.

In another series of experiments the influence of the glucose concentration of the afferent medium on the rate of growth was studied. The flow rates in these experiments was 20 ml per day per 10^6 cells initially. From Figure 2 it is seen that maximal rate of growth was obtained when the initial glucose concentration was 5 millimoles per litre. Higher initial glucose concentrations had no stimulatory effect on cell growth.

Thus, these results indicate that the optimal glucose concentration for growth of ELD-cells is 2–5 millimoles per litre.

In order to elucidate the need for serum in continuously perfused cultures the ELD-cells were grown in Eagle's Minimum Essential Medium modified as described above and fortified with 0–20 per cent foetal bovine serum. The gasphase contained 20 per cent oxygen and 5 per cent carbon dioxide. The pH of the medium in equilibration with the gasphase varied between 7.05 and 7.20. The initial cell number varied from 120 000–490 000 per flask. The experimental period was 3 days. The results are shown in Table 4 and Fig. 3. As it is seen serum was found to be essential for the growth of the ELD-cells also in the perfusion system. The optimal concentration was about 20 per cent.

In order to test whether the total supply of glucose or serum might be a limiting factor in stationary cultures of the ELD-cells a comparison of their growth was made in media containing 5 and 25 millimoles of glucose per litre and 0, 5, 10, 20 and 40 per cent serum, respectively. The media were changed daily, and the cultures were gassed with a mixture containing 5 per cent carbon dioxide, 20 per cent oxygen and 75 per cent nitrogen. Cell growth was assessed either by cell counts as previously described or by protein determinations using the Folin-

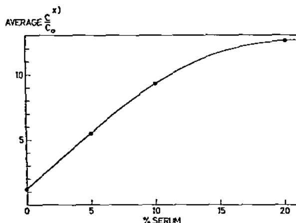


Fig 3

Relationship between serum content of medium and growth of ELD cells in a perfusion system

* Average rate of growth C_0 = initial cell concentration C = final cell concentration after 3 days of incubation

TABLE 4

Growth of ELD Cells under Continuous Perfusion with Serumfree Medium

	Control medium containing 20% foetal bovine serum	Serumfree medium
Number of experiments	16	16
Growth $\frac{C}{C_0}$ *	11 0 ± 1 1	0 89 ± 0 26

* Average rate of growth with 95 per cent confidence interval C_0 = initial cell concentration C = final cell concentration after 3 days of incubation
ELD see Table 1

Ciocalteau reaction as described by *Oyama & Eagle* (4) The results are shown in Tables 5 and 6 and in Fig 4 As it is seen the increase of the glucose concentration from 5 to 25 millimoles per litre had no stimulatory effect on cell growth The optimal serum concentration was 20 per cent (see Fig 4), while 40 per cent had a moderate inhibitory effect as shown in Table 6

DISCUSSION

Several systems for continuous perfusion have been described by previous authors The first experiment of this kind was carried out in 1927 by de *Haan* (5), who studied the morphology of leucocytes and macrophages grown on coverslips placed in a chamber through which the medium was passed by the application of a constant pressure

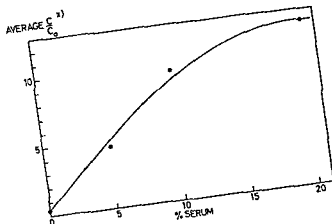


Fig 4

Relationship between serum content of medium and growth of FLD cells in stationary cultures

• Average rate of growth C_0 = inital cell concentration
C = final cell concentration after 3 days of incubation

TABLE 5

Growth of ELD Cells in Medium Containing 5 and 25 Millimoles of Glucose per Liter in Stationary Cultures

Initial cell mass	Exp period (days)	Growth $\frac{C}{C_0}$ 5 mM	25 mM	% of 25 mM	Number of observ
320 000 ± 36 000 cells	4	8 8 ± 2 4	12 0 ± 1 4	136	10
285 000 ± 120 000 cells	5	34 4 ± 5 1	29 2 ± 4 2	85	10
1 050 000 ± 310 000 cells	3	3 3 ± 0 4	3 8 ± 0 6	116	10
0 74 ± 0 05 mg protein	3	2 58 ± 0 29	2 43 ± 0 36	91	10
1 17 ± 0 13 mg protein	3	2 14 ± 0 38	1 93 ± 0 26	90	8
0 33 ± 0 04 mg protein	3	5 52 ± 0 30	4 94 ± 0 30	90	10

Average rate of growth with 95 per cent confidence interval
C_0 = initial cell concentration C = final cell concentration

FLD see Table 1

TABLE 6

Growth of ELD Cells in Stationary Cultures in Medium Containing 20 and 40 per Cent Fetal Bovine Serum

Initial cell mass (C_0)	Exp period (days)	Growth $\frac{C}{C_0}$ 20%	40%	% of 20%	Number of exp
320 000 ± 36 000 cells	4	8 8 ± 2 4	7 6 ± 1 1	86	10
1 050 000 ± 310 000 cells	3	3 3 ± 0 4	1 6 ± 0 5	49	10
0 74 ± 0 05 mg protein	3	2 58 ± 0 29	1 27 ± 0 28	49	10

ELD see Table 1

Better conditions for the microscopic examination of the living cultures were obtained in Pomerat s perfusion chamber (6) in which the tissue was growing between two coverslips forming the top and bottom of the chamber The same principle was used in the *Rose* chamber (7) which was a sandwich of steel plates coverslips and a rubber gasket These perfusion chambers were suitable for morphological studies but the amount of cell material cultivated was usually too small for biochemical work

Perfusion systems for the cultivation of human bone marrow have been designed by *Osgood & Muscovit* (8) and later by *Plum* (9) In both cases the medium was separated from the culture vessels by a semipermeable membrane and it was gassed with atmospheric air to provide oxygen to the cells and remove carbon dioxide The cells were growing in suspension

The application of perfusion systems with a high degree of automation used for mass cultivation of bacteria became possible when *Owens et al* (10) *Earle et al* (11) and others succeeded in culturing mammalian cells in suspension sultures Adapting the principles of the chemostates developed by *Novick* (12) and *Monod* (13) a steady state was aimed at by removing cells simultaneously with the removal of the medium at a rate corresponding to that of cell multiplication so that the cell concentration remained constant A simplified perfusion system of this type was described by *Cohen & Eagle* (14) and similar principles from the basis of the cytogenerator designed by *Graff & McCarty* (15) and of *Bjorklund s cytostate* (16) In the latter apparatus the medium flow was controlled automatically by the cell concentration registered photometrically Systems for cultivation of cells in suspension have a rather restricted use in the sense that only a few cell lines and no primary cultures are able to grow in suspension

A combination of the methods for monolayer cultures and cell suspensions was utilized by *McCoy et al* (17) in a perfusion apparatus containing glasshelices as a matrix for cell growth allowing three dimensional growth In this system excellent growth of Jensen sarcoma cells was obtained

The perfusion system described in the present paper was designed for the cultivation of cells in monolayer cultures equally suitable for biochemical and cytological studies Furthermore short distances of diffusion through the liquid phase were aimed at in order to enable satisfactory control of pO and pCO and thereby also of pH

In this system the ELD-cells were found to grow up to 3 times more rapidly than in stationary cultures By determination of the glucose concentration of the efferent medium the perfusion rate could easily be adjusted to meet the requirements for maximal growth

The reason for the higher rate of growth in the perfusion system was not revealed by the present experiments Neither the total supply

of glucose nor that of serum seemed to be the limiting factor Other components of the medium may be responsible but factors like pO_2 pCO_2 or pH might be of equal or greater importance

SUMMARY

An apparatus for cultivation of monolayer cultures in vitro with a continuous perfusion of medium and gas is described In this apparatus growth of FLD cells is found to be up to 3 times more rapid than in stationary cultures

REFERENCES

1 *Eagle H* Amino acid metabolism in mammalian cells cultures Science *130* 432 1959
2 *Dean R B & Dixon W J* Simplified statistics for small numbers of observations Analyt Chem 23 636 1951
3 *Horn H D & Bruns F H* Quantitative Bestimmung von L(+) Milchsaure mit Milchsauredehydrogenase Biochim Biophys Acta *21* 378 1956
4 *Oyama V I & Eagle H* Measurement of cell growth in tissue culture with a phenol reagent (Folin Ciocalteau) Proc Soc Exp Biol Med *91* 305 1956
5 *de Haan J* *Die* Umwandlung von Wanderzellen in Fibroblasten bei der Gewebezuchtung *in vitro* Arch Exp Zellforschung *3* 219 1927
6 *Pomerat C M* Perfusion chamber Methods in Medical Research *4* 275 1951
7 *Rose G* A separable and multipurpose tissue culture chamber Texas Rep Biol Med *12* 1074 1954
8 *Osgood E F & Muscovit A N* Culture of human bone marrow J A M A *106* 1888 1936
9 *Plum C M* *In vitro* study of bone marrow I A method for marrow culture Blood Spec Issue no 1 p 33 1947
10 *Owens O V H Gey G O & Gey M K* A new method for the cultivation of mammalian cells suspended in agitated fluid medium Proc Am Ass Cancer Research *1* 41 1953
11 *Earle W R Bryant J C & Schilling F L* Certain factors limiting the size of the tissue culture and the development of massive cultures Ann New York Acad Sci *58* 1000 1954
12 *Novick A & Szilard L* Description of the chemostat Science *112* 715 1950
13 *Monod J* La Technique de Culture Continue Théorie et Applications Ann Inst Pasteur *79* 390 1950
14 *Cohen E P & Eagle H* A simplified chemostat for the growth of mammalian cells Characteristics of cell growth in continuous culture J Exp Med *113* 467 1961
15 *Graff S & McCarty K S* Sustained cell culture Exp Cell Research *13* 348 1957
16 *Bjorklund B Bjorklund V & Paulsson J F* Automatic cell culture Proc Soc Exp Biol Med *108* 385 1961
17 *McCoy T A Whittle W & Conway E* A glass helix perfusion chamber for massive growth of cells *in vitro* Proc Soc Exp Biol Med *109* 235 1962

Acta path et microbiol scandinav 66, 41–45, 1966

The Gade Institute, Department of Pathology, The University of Bergen Norway
(Head Professor E Waaler, M D)

THE EFFECT OF VARIOUS MAMMALIAN SERA ON THE ONCOLYSIS OF BERGEN A4 ASCITES CARCINOMA CELLS BY HUMAN SERUM

By

F Hartveit[1]

Received 22 x 65

It has recently been shown that the process of oncolysis of mouse tumour cells by human serum is qualitatively similar to that of immune haemolysis (5) The tumour cells are thought to have been sensitized in the host mouse (1) and the human serum is said to supply only complement to the system (6) It has also been shown that mouse tumour ascitic fluid can inhibit such immunological lysis (2) and that mouse serum inhibits the lysis of mouse tumour cells by mouse complement (8)

The present study was undertaken to see whether the inhibitory action of mouse serum on the oncolytic reaction is parallelled by other mammalian sera—i e to see if the effect is merely physical or whether a species specificity is involved As repeated tests have given similar results only specimen experiments will be quoted here

MATERIAL AND METHODS

The tumour used was the Bergen A4 mouse ascites carcinoma (3) kept by serial transplantation in strain A/Sn mice Tumour cells from a 15 day transplant and from a 16-day transplant both in female mice were used Blood was obtained from 5 blood donors and 5 cancer patients It was allowed to clot at room temperature The clot was spun down gently and the supernatant serum stored within 3 hours of bleeding at —20° C Before use it was thawed quickly at 37° C. Goat guinea pig rabbit rat and mouse (A/Sn and our closed colony) serum were obtained and treated in a similar way

The tumour cell suspensions used in all experiments consisted of one part whole tumour ascites in 20 parts physiological saline Suspensions were made up just before use The sera were used whole

Experimental Procedure

The oncolytic action of the 10 human sera was tested using one part saline one part serum and one part tumour cell suspension from the 15 day transplant Wet preparations were set up as described previously (1) at 20° C and examined for oncolysis at intervals of 15 minutes for one hour

The oncolytic activity of the other sera was tested in the same way

[1] Research Fellow Norwegian Cancer Society

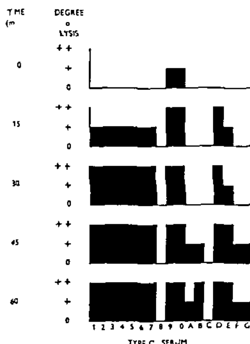


Fig 1

The oncolytic activity of human serum (1 10) on Bergen A4 ascites carcinoma cells compared to that of goat (A) guinea pig 1 (B) and 2 (C) rabbit (D) rat (E) and mouse A/Sn (F) and closed colony (G) Note control cells in saline negative
++ complete lysis + some lysis 0 no lysis

Tests for inhibitory effect on the oncolytic reaction Each of the 10 human sera was first retested for oncolytic activity against tumour cells from the 16 day trans plant All the other sera were then tested against each of the 10 human sera using one part human serum one part of the other serum and one part of the tumour cell suspension Wet preparations were set up as above and readings taken at 15 minute intervals for half an hour

Control cells were set up in saline in each case

RESULTS

The oncolytic action of the human sera is shown in Fig 1 where it is compared to that of *the other sera* All degrees of oncolytic activity were found from complete lysis by 15 minutes to complete lack of lysis after one hour

Tests for inhibiting effect on the oncolytic reaction are shown in Fig 2 First the basic oncolytic action of the 10 human sera was reassessed using the new tumour cell suspension The results were similar to those on the preceeding day Next the Fig shows the result of adding each serum in turn to the human sera It was found that in the majority of cases the addition of two sera led to an increase in the oncolytic activity This potentiating effect was marked with goat serum both oncolytic and non oncolytic guinea pig serum and

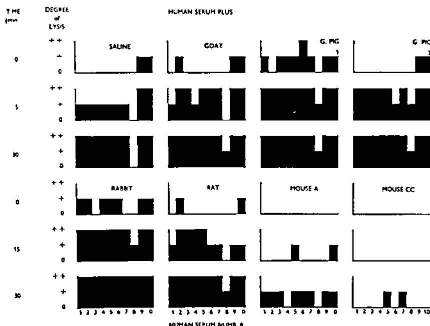


Fig 2

The effect of addition of sera of different species on the lytic action of human serum on Bergen A4 ascites carcinoma cells Note control cells in saline negative ++ complete lysis + some lysis 0 no lysis

rabbit serum Rat serum potentiated the action of human sera nos 2, 3, 4 and 5 but there was a suggestion of inhibitory action with sera 9 and 10 Both mouse sera, on the other hand, markedly inhibited the oncolytic action of human serum

Control preparations were uniformly negative

DISCUSSION

The present experiment shows, as previous results had suggested (9, 7), that the type of complement used, within the limits of the experiment, is not important to the oncolytic reaction The sensitized tumour cells showed some degree of lysis with all types of sera tested

When two sera are mixed and added to sensitized tumour cells the oncolytic reaction could be effected in several ways The complement contents of the two sera might supplement each other, giving an additive effect, or conversely the complement factors might compete with each other, giving an anticomplementary effect On the other hand some other factor might block the oncolytic reaction at any point

Some of these possibilities can be evaluated from the present work The results indicate that an additive effect is usual when two sera are mixed Even when a serum is non oncolytic on its own it may

potentiate the action of another serum For example serum from guinea-pig 2 was non-oncolytic—but it potentiated the action of 8 out of 10 human sera This suggests that guinea-pig 2 serum lacked enough of one or more of the complement components needed for oncolysis (5), but that the other components it contained were the limiting factors in the human sera Hence addition increased the total lytic effect Similar argumentation applies for goat, guinea-pig 1 and rabbit serum

On the other hand, when mouse serum is added to human serum the oncolytic reaction is markedly inhibited Thus mouse serum, as well as mouse tumour ascitic fluid (7) contains some factor(s) that is able to inhibit the lytic action of human complement on sensitized tumour cells, as well as the lytic action of mouse complement on mouse tumour cells Thus the inhibitor does not appear to show a species specificity as far as complement is concerned However, within the limits of the present experiment, it seems that the inhibitor in this system is confined to mouse serum, a possible exception being rat serum, which showed slight inhibitory effects with two human sera This specificity in distribution rules out the possibility of a purely physical effect due, for example, to the protein concentration of the medium

Where this inhibitory factor acts in the reaction leading to tumour cell lysis is not yet clear But some possibilities can now be ruled out It does not act at the stage of union of antigen and antibody as this stage was complete before the mouse serum was added to the system In addition tumour cells become progressively sensitized in vivo where their lysis is prevented by this inhibitory factor (1) Further it is unlikely that the inhibitor prevents the absorption of complement factor 1, as it has been shown that the C1 content of mouse serum drops during progressive tumour growth (4) Apart from this its mode of action is unknown, but it seems likely that it could be of an anticomplementary type Be this as it may it is clear that the action of both mouse complement and human complement is blocked by this inhibitor Thus the action of the inhibitor is not specific to the type of complement used However the present experiment suggests that there may be a species specificity between inhibitor and tumour cell or perhaps between inhibitor and antibody, or both

SUMMARY

The action of various mammalian sera was tested in an oncolytic system using sensitized BA4 ascites carcinoma cells The majority of the sera tested showed some degree of lysis The degree of lysis was increased when human serum was mixed with goat, guinea pig or rabbit serum but the addition of mouse serum, and to a much lesser extent rat serum, inhibited the oncolytic action of human serum

These results suggest a species specificity between the inhibitor and either the tumour cells and or their sensitizing antibody

REFERENCES

1 *Hartveit F* The lytic action of fresh human serum on Bergen A4 ascites carcinoma cells Brit J Cancer *18* 721 725 1964
2 *Hartveit F* The demonstration of an inhibitor of oncolysis in the ascitic fluid from the Ehrlich ascites carcinoma Brit J Cancer *18* 726–729 1964
3 *Hartveit F* The significance of the blood content of the Bergen A4 mouse ascites carcinoma Brit J Cancer *18* 557 563 1964
4 *Hartveit F* The complement content of the serum of normal as opposed to tumour bearing mice Brit J Cancer *18* 714 720 1964
5 *Hartveit F* A comparison of the processes of immune haemolysis and oncolysis J Path & Bact *89* 145–151 1965
6 *Hartveit F* An analysis of the reactants in the oncolytic reaction J Path & Bact *89* 551 555 1965
7 *Hartveit F* Inhibition of lysis of sensitised tumour cells in the presence of complement Brit J Cancer *19* 589 593 1965
8 *Hartveit F* *In vitro* oncolysis and its inhibition by mouse serum Acta path et microbiol scandinav *65* 354–358 1965
9 *Kuru M Kosaki G Itou i H Isibasi H & Tokunaga A* The mechanism of cytolysis of Ehrlich ascites tumour brought into contact with normal human serum Acta Un Internat contra Cancer *15* 165 170 1959

Acta path et microbiol scandinav 66 46–50 1966

The Gade Institute, Department of Pathology The University of Bergen Norway
(Head Professor F Waaler, M D)

IN VITRO POTENTIATION OF ONCOLYSIS BY BOVINE ALBUMIN

By

F HARTVEIT[1]

Received 22 v 65

The morphology of ascitic tumour cells differs in different concentrations of bovine albumin (3) High concentrations of bovine albumin in physiological saline have been shown to hinder (osmotically) the lysis of such cells by heterologous antisera (2) The morphological changes in the tumour cells in lower albumin concentrations suggest that these concentrations also might hinder the oncolysis of such cells by complement—i e the oncolytic reaction This idea is seconded by the finding that similar morphological changes occur in late transplants in vivo when copious amounts of tumour ascitic fluid, which is rich in albumin (5), are present

This possibility was investigated using tumour cells from the Bergen A4 ascites carcinoma As similar results have been obtained repeatedly only specimen experiments are reported

MATERIAL AND METHODS

The tumour cells came from 15 16 and 20 day transplants of the Bergen A4 carcinoma in female A/Sn mice Crystalline bovine albumin (fraction 5) was obtained from Armour pharm Co Ltd A 20 per cent stock solution was made up in sterile physiological saline Other concentrations were made up from this as required The human sera came from 5 blood donors and 25 cancer patients The sera were collected and stored as reported previously (4) Serum from rats rabbits guinea pigs and mice (A/Sn and the closed colony kept here) were obtained and treated in a similar way

The tumour cells were used as a 1 in 20 suspension of whole tumour ascites in either physiological saline or a 2 5 per cent bovine albumin solution Cell suspensions were made up just before use The sera were used whole

Tests for oncolytic activity in the presence of bovine albumin were carried out on 5 *donor sera and 5 patient sera* The reactants (1 1 1) were a) serum saline and tumour cell suspension (in saline) and b) serum 2 5 per cent bovine albumin solution and tumour cell suspension (in saline)

Similar tests were carried out on the *other animal sera* The reactants (1 1 1) were a) serum saline and tumour cell suspension (in saline) and b) serum saline and tumour cell suspension (in albumin)

Tests for oncolytic activity with different proportions of bovine albumin were carried out on 10 other patient sera The reactants (1 1 1) were a) serum saline and tumour cell suspension (in saline) b) serum saline and tumour cell suspension

[1] Research Fellow Norwegian Cancer Society

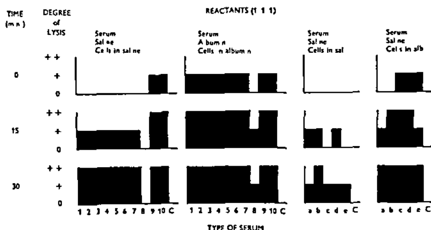


Fig 1

The oncolytic activity of human serum (1–10) and of serum from rat (a), rabbit (b) guinea pig (c), mouse, strain A/Sn (d) and closed colony (e) in the presence of bovine albumin (see text) (C control without serum)
++ complete lysis + some lysis 0 no lysis

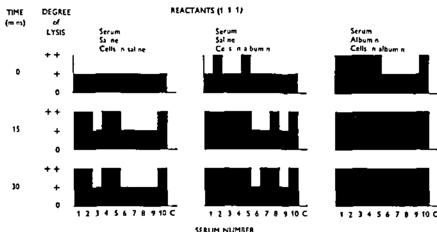


Fig 2

Increasing oncolytic activity of human sera (1 10) with increasing proportions of albumin (C control without serum)
++ complete lysis + some lysis 0 no lysis

(in albumin) and c) serum 2 5 per cent albumin solution and tumour cells suspension (in albumin)

The oncolytic activity in increasing concentrations of bovine albumin was tested with the 10 further patient sera The reactants (1 1 1) were a) one part serum one part saline and one part tumour cell suspension (in saline) b) as above with 2 5 per cent albumin in place of the one part saline c) with 5 per cent albumin d) with 10 per cent and e) with 20 per cent albumin in place of the one part saline

Control preparations were run in all tests with cells in saline and the different albumin concentrations

REFFRENCI S

4 *Harris A* & *Moore D H* The anticomplementary
ı ı m gamma globulin J Immunol 49 223 233 1944
& *Goldberg B* The effect of antibody and complement on
ntrol in ascites tumour cells and erythrocytes J exp Med
1959
gnificance of the protein content of Ehrlich s ascites carcinoma
t microbiol scandinav 59 51 62, 1963
omparison of the processes of immune haemolysis and onco-
Path & Bact 89 145 151, 1965
alılay P & *Williams Ashman H G* Studies on the Ehrlich ascites
o r Cancer Res 11 855-863, 1951

Acta path et microbiol scandinav 66 51–54 1966

The Gade Institute, Department of Pathology The University of Bergen Norway
(Head Professor F Waaler, M D)

IN VITRO POTENTIATION OF ONCOLYSIS BY RHEOMACRODEX

By

F HARTVEIT[1]

Received 22 v 65

It has recently been shown that lysis of sensitized tumour cells in the presence of complement—*i e* the oncolytic reaction—is potentiated in the presence of bovine albumin (6) The use of albumin in this respect can perhaps be likened to its use in tests for incomplete antibody This in turn suggests that other substances, such as the dextrans, might also potentiate the oncolytic reaction

This hypothesis was tested and the results are demonstrated in the following experiments

MATERIAL AND METHODS

Tumour cells from a 15 day transplant of the *Ehrlich ascites carcinoma* in a female mouse of the closed colony used at this Institute (2) and tumour cells from a 15 day transplant of the *Bergen A4 ascites carcinoma* in a female A/Sn mouse were used Tumour cell suspensions were made up in physiological saline using one part whole Ehrlich ascites in 10 of saline and one part whole Bergen A4 ascites in 20 of saline Suspensions were made up just before use

Human serum was obtained from 10 cancer patients and stored as described previously (4) The sera were used whole

Rheomacrodex (mol wt 40 000) was used as the test fluid

The standard 10 per cent solution in physiological saline was further diluted to give 2 5 and 5 per cent solutions also

Experimental Procedure

Oncolysis of Ehrlich ascites carcinoma cells in saline and in 2.5 per cent rheomacrodex a) One part of tumour cell suspension was put up against one part of each of the 10 sera in turn plus two parts of saline b) as above but with two parts of 2 5 per cent rheomacrodex replacing the two parts of saline

Controls were set up in which the sera were omitted

Oncolysis of Bergen A4 ascites carcinoma cells a) One part of tumour cell suspension was set up with one part of each of the sera in turn plus one part of saline b) as above using one part 2 5 per cent c) 5 per cent and d) 10 per cent rheomacrodex in place of one part saline

Controls were set up in which the sera were omitted

Wet preparations were made and set up as described previously (4) at 20° C They were examined for oncolysis at 15 minute intervals for 1 hour

[1] Research Fellow Norwegian Cancer Society

REFERENCES

Ha... & Moore D H: The ...
... nm
... Ilb
ol in

...icance of the protein content of Ehrlich's ascites carcinoma
...icrobiol scandinav 59 51–62, 1963
...parison of the processes of immune haemolysis and onco-
... Bact 89 145–151, 1965
... P & Williams Ashman H G: Studies on the Ehrlich ascites
... Res 11 855–863, 1951

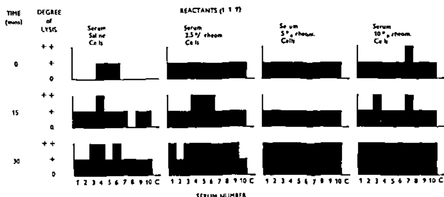


Fig 2

The oncolytic action of 10 human sera on Bergen A4 ascites carcinoma cells in saline and in increasing concentration of rheomacrodex (C control without serum) ++ complete lysis + some lysis 0 no lysis

Thus with both types of cells rheomacrodex was able to increase both the speed and the extent of the oncolytic reaction

DISCUSSION

The present results show that the oncolytic action of human sera on cells from both Ehrlich ascites carcinoma and the Bergen A4 ascites carcinoma may be potentiated by the addition of rheomacrodex to the system This finding is similar to that previously reported (6)—*i e* that the addition of bovine albumin to Bergen A4 ascites carcinoma cells may potentiate oncolysis Furthermore the finding that the higher concentrations of bovine albumin may give transient inhibition before their potentiation of the reaction becomes evident, is repeated here—though it is less marked—with rheomacrodex

While these findings do not answer any questions they raise many problems, and open up new perspectives

The Ehrlich ascites carcinoma cells used were homografted cells and as such can be expected to be sensitized by antibody from their host mouse (5) The Bergen A4 ascites carcinoma cells were isografted -but have also been shown to be sensitized by antibody supplied by the otherwise genetically compatible host to tumour specific antigen (3) In both cases the human serum supplies complement to the system In both cases the sensitized cells lyse in vitro in the presence of complement and in both cases the degree of lysis may be increased by the addition of either bovine albumin or rheomacrodex These two substances are both known to unmask the presence of incomplete antibody (see 8) Is this what is happening here? Both substances have one physical characteristic in common their high molecular

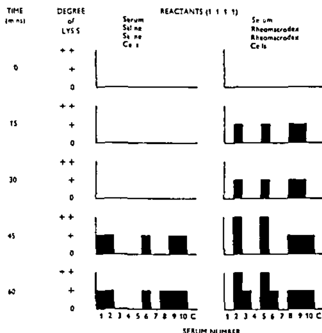


Fig 1

The oncolytic action of 10 human sera on Ehrlich ascites carcinoma cells in saline and in 2 5 per cent rheomacrodex (C control without serum) ++ complete lysis + some lysis 0 no lysis

RESULTS

The extent of the oncolytic reaction with Ehrlich cells in saline and in 2 5 per cent rheomacrodex is shown in Fig 1 At the dilutions used the human sera gave a minimum of lysis in saline However, replacement of saline by rheomacrodex led to potentiation in 7 cases, no change in 2 and inhibition in one case (no 1)

The extent of the oncolytic reaction with Bergen A4 cells is shown in Fig 2 All the sera were lytic at the dilutions used The addition of 2 5 rheomacrodex gave increased lysis with 7 sera in the immediate preparations and increased lysis with 2 more at 15 minutes The final serum was highly lytic and its ability was not potentiated 5 per cent rheomacrodex gave a similar immediate potentiation of 7 sera and gave complete lysis with all sera by 30 minutes However at 15 minutes there was less lysis with 3 of the sera than with 2 5 per cent, and in serum no 4 lysis was less than with saline The 10 per cent solution potentiated 7 out of 10 sera in the immediate preparations, leaving the other 3 unchanged, *i e* as with saline At 15 minutes lysis was more pronounced in 9 out of 10 In no 4 there was less lysis By 30 minutes there was complete lysis in all cases

Control preparations were uniformly negative

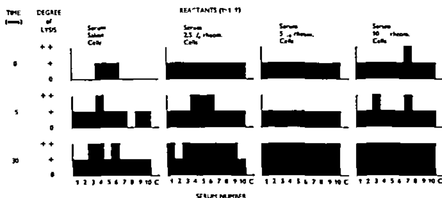


Fig 2

The oncolytic action of 10 human sera on Bergen A4 ascites carcinoma cells in saline and in increasing concentration of rheomacrodex (C control without serum) ++ complete lysis + some lysis 0 no lysis

Thus with both types of cells rheomacrodex was able to increase both the speed and the extent of the oncolytic reaction

DISCUSSION

The present results show that the oncolytic action of human sera on cells from both Ehrlich ascites carcinoma and the Bergen A4 ascites carcinoma may be potentiated by the addition of rheomacrodex to the system This finding is similar to that previously reported (6)—*i e* that the addition of bovine albumin to Bergen A4 ascites carcinoma cells may potentiate oncolysis Furthermore the finding that the higher concentrations of bovine albumin may give transient inhibition before their potentiation of the reaction becomes evident is repeated here—though it is less marked—with rheomacrodex

While these findings do not answer any questions they raise many problems and open up new perspectives

The Ehrlich ascites carcinoma cells used were homografted cells and as such can be expected to be sensitized by antibody from their host mouse (5) The Bergen A4 ascites carcinoma cells were isografted but have also been shown to be sensitized by antibody supplied by the otherwise genetically compatible host to tumour specific antigen (3) In both cases the human serum supplies complement to the system In both cases the sensitized cells lyse in vitro in the presence of complement and in both cases the degree of lysis may be increased by the addition of either bovine albumin or rheomacrodex These two substances are both known to unmask the presence of incomplete antibody (see 8) Is this what is happening here? Both substances have one physical characteristic in common—their high molecular

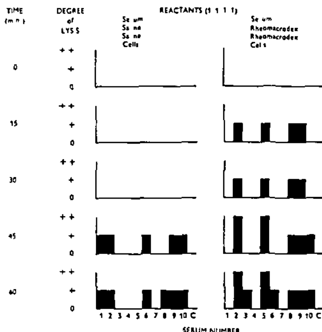


Fig 1

The oncolytic action of 10 human sera on Ehrlich ascites carcinoma cells in saline and in 2 5 per cent rheomacrodex (C control without serum) ++ complete lysis + some lysis 0 no lysis

RESULTS

The extent of the oncolytic reaction with Ehrlich cells in saline and in 2 5 per cent rheomacrodex is shown in Fig 1 At the dilutions used the human sera gave a minimum of lysis in saline However, replacement of saline by rheomacrodex led to potentiation in 7 cases, no change in 2 and inhibition in one case (no 1)

The extent of the oncolytic reaction with Bergen A4 cells is shown in Fig 2 All the sera were lytic at the dilutions used The addition of 2 5 rheomacrodex gave increased lysis with 7 sera in the immediate preparations and increased lysis with 2 more at 15 minutes The final serum was highly lytic and its ability was not potentiated 5 per cent rheomacrodex gave a similar immediate potentiation of 7 sera and gave complete lysis with all sera by 30 minutes However at 15 minutes there was less lysis with 3 of the sera than with 2 5 per cent, and in serum no 4 lysis was less than with saline The 10 per cent solution potentiated 7 out of 10 sera in the immediate preparations, leaving the other 3 unchanged, *i e* as with saline At 15 minutes lysis was more pronounced in 9 out of 10 In no 4 there was less lysis By 30 minutes there was complete lysis in all cases

Control preparations were uniformly negative

Acta path et microbiol scandinav 66 55–61, 1966

The Gade Institute Department of Pathology The University of Bergen, Norway
(Head Professor F Waaler, M D)

IN VITRO ABROGATION OF THE INHIBITORY ACTION OF TUMOUR ASCITIC FLUID ON THE ONCOLYTIC REACTION

By

F Hartveit[1]

Received 22 v 65

Evidence is collecting from work with two transplantable non spe cific mouse ascites carcinomas—the Ehrlich ascites carcinoma and the Bergen A4 ascites carcinoma—that these tumour cells survive in vivo not because the host fails to put up an immunological defence against them—but because this attack fails to achieve its objective—i e tumour cell lysis (6) Thus a form of immunological tolerance, of the type specified by *Medawar* (11) as "states in which there is some reason to believe that immunological activity has been thwarted' is established In the case of the Ehrlich ascites carcinoma and with homografted Bergen A4 ascites carcinoma this tolerance is to homo-grafted tissue—but with isologous transplants of the Bergen A4 ascites carcinoma tolerance is established to tumour tissue as such

This state of immunological tolerance is mediated via a normal constituent of mouse serum (7), which is therefore said to contain an inhibitor(s) of immunoligical tumour cell lysis The antibody in these systems is probably supplied by the host mouse (2, 3, 4, 5) Thus the inhibitor prevents the lysis of mouse tumour cells sensitized by mouse antibody

It has recently been shown that bovine albumin and rheomacrodex potentiate the action of complement on mouse sensitized mouse tumour cells (9, 10) In vitro studies were carried out to see if this potentiation of oncolysis occurred in the presence of inhibitor, i e in the presence of the factor(s) that mediates immunological tolerance *in vivo* As similar results have been obtained repeatedly only "demonstration runs" will be presented here

MATERIAL AND METHODS

Serum from 30 blood donors was obtained and stored fresh at —20° C as described previously (8) as was goat guinea pig rat rabbit and mouse (A/Sn and our closed colony) serum

[1] Research Fellow Norwegian Cancer Society

Tumour cells from 15 16 and 20 day transplants of the Bergen A4 ascites carcinoma in female A/Sn (*i e* isologous) mice were used

Tumour ascitic fluid (cell free) was obtained from the tumour transplants by centrifuging half of the tumour ascites The fluid was used whole

Bovine albumin was obtained in the crystalline form (fraction 5) and used as a 2 5 per cent and 10 per cent solution in physiological saline

Rheomacrodex (mol wt 40 000) was used as a 2 5 per cent and 10 per cent solution in physiological saline

Tumour cell suspensions consisted of a one in 20 suspension of whole tumour ascites in physiological saline or in ascitic fluid or the albumin or rheomacrodex solutions

Experimental Procedure

1 The effect of *2 5 per cent bovine albumin* on oncolysis in the presence of ascitic fluid was tested using *10 donor sera* Three parallel series were set up the reactants (1 1 1) being saline—serum—cells in saline ascitic fluid—serum—cells in saline ascitic fluid—serum—cells in albumin as detailed in Fig 1

2 The *sera from other animals* were tested in the same way using the same parallel series see Fig 2

3 The effect of *2 5 per cent bovine albumin* on oncolysis in the presence of ascitic fluid was compared to that of *2 5 per cent rheomacrodex* using *10 other donor sera* Four parallel series were set up the reactants (1 1 1) being saline—serum—cells in saline saline—serum—cells in ascitic fluid rheomacrodex—serum—cells in ascitic fluid albumin—serum—cells in ascitic fluid as detailed in Fig 3

The albumin test here differs from 1) that the cells were suspended in ascitic fluid instead of saline *i e* the order of addition of reactants differs

4 The effect of *10 per cent bovine albumin* and *10 per cent rheomacrodex* on oncolysis in the presence of ascitic fluid was tested using *10 further donor sera* Four parallel series were set up the reactants (1 1 1) being saline—serum cells in saline ascitic fluid—serum—cells in saline ascitic fluid—serum—cells in rheomacrodex ascitic fluid—serum—cells in albumin as detailed in Fig 4

Control preparations in which sera were omitted were run in each case

Wet preparations were made up as described previously (7) at 20° C and examined for oncolysis at 15 minute intervals for 30 minutes in exp 1 and for 1 hour in the others

RESULTS

1 Fig 1 shows that the oncolytic action of *human sera* was decreased in the presence of ascitic fluid When albumin was added to the system this inhibitory action was counteracted immediately with 4 sera after 15 minutes with one after 30 minutes with 4 and remained unchanged with one (no 3)

2 Fig 2 shows that the oncolytic ability of *serum from other animals* was also reduced in the presence of ascitic fluid When albumin was added the activity of rat and rabbit serum returned and was indeed potentiated That of goat serum was restored in part while that of guinea pig serum and mouse serum was not restored

3 The findings with 2 5 per cent solutions of *rheomacrodex* and *albumin* and *10 donor sera* are shown in Fig 3 The lytic activity of 8 out of 10 sera was reduced in the presence of ascitic fluid—that of the other two remained unchanged When rheomacrodex was added the activity in the immediate preparation of 3 of the sera was restored to that in saline and that of 3 others potentiated At 15 minutes the activity of 3 more was restored one remained unchanged At 30 minutes the results were the same as in ascitic fluid By 1 hour the activity

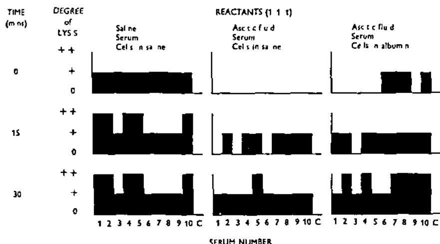


Fig 1

The effect of 2 5 per cent bovine albumin on the oncolysis of Bergen A4 ascites carcinoma cells by 10 human sera in the presence of tumour ascitic fluid (C control without serum)
++ complete lysis + some lysis 0 no lysis

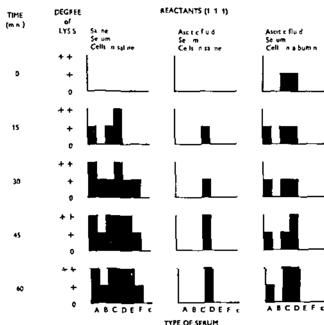


Fig 2

The effect of 2 5 per cent bovine albumin on the oncolysis of Bergen A4 ascites carcinoma cells by goat (A) guinea pig (B) rat (C) rabbit (D) and mouse A/Sn (E) and closed colony (F) serum in the presence of tumour ascitic fluid (C control without serum)
++ complete lysis + some lysis 0 no lysis

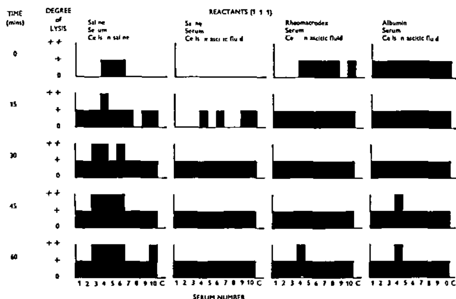


Fig 3

The effect of 2·5 per cent bovine albumin and 2·5 per cent rheomacrodex on the oncolysis of Bergen A4 ascites carcinoma cells by 10 human sera in the presence of tumour ascitic fluid (C control without serum)
++ complete lysis + some lysis 0 no lysis

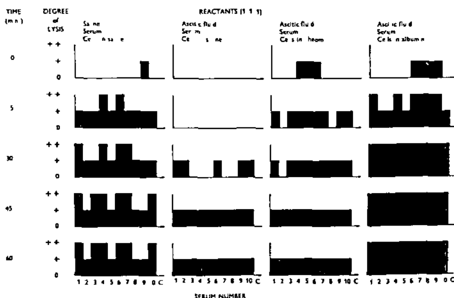


Fig 4

The effect of 10 per cent rheomacrodex and 10 per cent bovine albumin on the oncolysis of Bergen A4 ascites carcinoma cells by 10 human sera in the presence of tumour ascitic fluid (C control without serum)
++ complete lysis + some lysis 0 no lysis

of serum 4 in rheomacrodex had returned to what it was in saline the others remained as in ascitic fluid The addition of albumin restored the activity of 3 of the sera in the immediate preparation and potentiated that of the other 7 At 30 minutes the results were similar to those in rheomacrodex and ascitic fluid At one hour the results were like those in rheomacrodex

Thus in both cases the speed but not the extent of the reaction has been increased

4 The findings with *10 per cent solutions of rheomacrodex and albumin* and *10 other donor sera* are demonstrated in Fig 4 The lytic ability of all 10 sera was reduced in the presence of ascitic fluid for example no lysis occurred in the presence of ascitic fluid at 15 minutes while all the sera gave lysis at this time in its absence When rheomacrodex was added the lytic ability was restored at 15 minutes in 8 out of 10 cases and that of 3 of these sera was potentiated However the final lytic result in rheomacrodex was no greater than that in ascitic fluid Thus the reaction has been speeded up but not increased in extent When albumin was added the activity of 9 out of 10 sera was greater than that in saline—*i e* not only has the action of the inhibitor been overcome but the original oncolytic activity has been potentiated The action of the other serum was completely restored

All control preparations were uniformly negative

DISCUSSION

The present experiments demonstrate that tumour ascitic fluid can decrease the lytic action of human serum and of other sera on tumour cells from the Bergen A4 mouse ascites carcinoma A 2 5 per cent solution of bovine albumin may be able to counteract this inhibition Further 2 5 per cent solutions of both rheomacrodex and albumin may even speed up the reaction in the presence of tumour ascitic fluid—and albumin may increase its extent The same is true of 10 per cent as of 2 5 per cent rheomacrodex but 10 per cent albumin both speeds up and potentiates the oncolytic reaction in the presence of ascitic fluid The order of addition of reactants does not seem to be critical to the results

Thus these *in vitro* experiments confirm the inhibitory action of tumour ascitic fluid on mouse tumour cells sensitized by mouse antibody (6) They demonstrate within the limits of the experiment that this inhibitory action is not dependent on the type of complement used Further the experiments show that both bovine albumin and to a certain extent rheomacrodex can potentiate the lytic action of complement in the presence of inhibitor of oncolysis (*i e* tumour ascitic fluid) as well as in its absence (9 10)

The action of this inhibitor has been shown to be specific to either the antigen and/or the antibody in this system (8) At first sight the

potentiating action of albumin appears to show a similar specificity as it failed to restore the oncolytic activity of mouse serum However, as mouse serum also contains this inhibitor of oncolysis (7) this is not a fair comparison In addition, as albumin also failed to restore the activity of poorly lytic guinea pig serum, these findings as a whole stress that the balance between complement and inhibitor is decisive to the outcome of the reaction, rather than any specificity between the type of complement and the potentiating action of bovine albumin This interpretation is in keeping with the previous report (9) that bovine albumin potentiates the action of complement—irrespective of type and including mouse complement—in the absence of inhibitor

Such an interpretation of the findings—in short that bovine albumin acts in a non-specific manner by potentiating the action of complement, would bring these findings into line with the previous findings in the haemolytic system In 1944 *Davis et al* reported that purified gamma globulin is highly anticomplementary and that its anticomplementary action was counteracted by the addition of albumin to the system They indeed concluded, "The complement fixation reaction as generally carried out on whole serum is therefore possible only because of the inhibitory effect of the other serum proteins upon the anticomplementary action of this component" (gamma globulin) In other words they showed that albumin could potentiate the action of complement on sensitized cells in the presence of an inhibitor of immunological lysis—a situation parallel to that in the present work Viewed in this light it seems that the potentiation of oncolysis seen in the absence of inhibitor may be due to albumin counteracting the anticomplementary effect of the native gamma globulin in the serum supplying the complement

This raises the question as to whether the inhibitor, which is known to be a normal constituent of mouse serum (7), may not be gamma globulin *Davis et al* (1) have shown that gamma globulin can act as an inhibitor of immunological lysis in a heterologous system The present experiment suggests that it may also act in an isologous system But as *Davis et al* point out the inhibitory action of gamma globulin in their system is usually cancelled out by the presence of serum albumin *This is not so in the present isologous system*, as the inhibitor acts in whole serum (7) The explanation here may be that gamma globulin has a greater affinity for the immune complex, *i e* antigen—antibody—complement, in an isologous system than in a heterologous system, and as a result is more anticomplementary in the former

It is tentatively suggested that such a mechanism, with its resultant immunological tolerance may be responsible for the maintenance of immunological homeostasis—*i e* the survival of self tissue in an organism with an immune apparatus If this is so the present concept of autoimmune disease may need revision and the failure of the body's

immunological defences to prevent the growth of cancer tissue might also be explained

SUMMARY

Bovine albumin and to a lesser extent, rheomacrodex potentiate the action of complement on sensitized mouse tumour cells in the presence of an inhibitor(s) of immunological lysis in tumour ascitic fluid, in vitro The possibility that this inhibitor, which is known to be a normal constituent of mouse serum, is gamma globulin and that its anticomplementary action is inhibited by bovine albumin is ventilated The implications of such a mechanism which would amount in vivo to a form of immunological tolerance, are discussed briefly

REFERENCES

1 *Davis B D., Kabat E A Harris A & Moore D H* The anticomplementary activity of serum gamma globulin J Immunol *49* 223 233 1944
2 *Hartveit F* The significance of the blood content of the Bergen A4 mouse ascites carcinoma Brit J Cancer *18* 557 563 1964
3 *Hartveit F* The lytic action of fresh human serum on Bergen A4 ascites carcinoma cells Brit J Cancer *18* 721 725 1964
4 *Hartveit F* A comparison of the processes of immune haemolysis and oncolysis J Path & Bact *89* 145–151 1965
5 *Hartveit F* An analysis of the reactants in the oncolytic reaction J Path & Bact *89* 551 555 1965
6 *Hartveit F* Inhibition of lysis of sensitised tumour cells in the presence of complement Brit J Cancer *19* 589 593 1965
7 t
8 n
9 *Hartveit F* *In vitro* potentiation of oncolysis by bovine albumin Acta path et microbiol scandinav *66* 46 50 1966
10 *Hartveit F* *In vitro* potentiation of oncolysis by rhemacrodex Acta path et microbiol scandinav *66* 51 54 1966
11 *Medawar P B* Theories of immunological tolerance In Cellular aspects of immunity p 134 Ciba Foundation Symposium London J & A Churchill Ltd 1960

potentiating action of albumin appears to show a similar specificity as it failed to restore the oncolytic activity of mouse serum However, as mouse serum also contains this inhibitor of oncolysis (7) this is not a fair comparison In addition, as albumin also failed to restore the activity of poorly lytic guinea pig serum, these findings as a whole stress that the balance between complement and inhibitor is decisive to the outcome of the reaction, rather than any specificity between the type of complement and the potentiating action of bovine albumin This interpretation is in keeping with the previous report (9) that bovine albumin potentiates the action of complement—irrespective of type and including mouse complement—in the absence of inhibitor

Such an interpretation of the findings—in short that bovine albumin acts in a non specific manner by potentiating the action of complement, would bring these findings into line with the previous findings in the haemolytic system In 1944 *Davis et al* reported that purified gamma globulin is highly anticomplementary and that its anticomplementary action was counteracted by the addition of albumin to the system They indeed concluded, "The complement fixation reaction as generally carried out on whole serum is therefore possible only because of the inhibitory effect of the other serum proteins upon the anticomplementary action of this component' (gamma globulin) In other words they showed that albumin could potentiate the action of complement on sensitized cells in the presence of an inhibitor of immunological lysis—a situation parallel to that in the present work Viewed in this light it seems that the potentiation of oncolysis seen in the absence of inhibitor may be due to albumin counteracting the anticomplementary effect of the native gamma globulin in the serum supplying the complement

This raises the question as to whether the inhibitor, which is known to be a normal constituent of mouse serum (7), may not be gamma globulin *Davis et al* (1) have shown that gamma globulin can act as an inhibitor of immunological lysis in a heterologous system The present experiment suggests that it may also act in an isologous system But as *Davis et al* point out the inhibitory action of gamma globulin in their system is usually cancelled out by the presence of serum albumin This is not so in the present isologous system, as the inhibitor acts in whole serum (7) The explanation here may be that gamma globulin has a greater affinity for the immune complex, *i e* antigen —antibody—complement, in an isologous system than in a heterologous system, and as a result is more anticomplementary in the former

It is tentatively suggested that such a mechanism, with its resultant immunological tolerance, may be responsible for the maintenance of immunological homeostasis—*i e* the survival of self tissue in an organism with an immune apparatus If this is so the present concept of autoimmune disease may need revision and the failure of the body s

together with the polysaccharides When the precipitate was dissolved in water again the proteins were insoluble and were removed by centrifugation The clear supernatant containing the polysaccharides after treatment with the liver extract was dialysed in running tap water for 24 hr and reprecipitated with 3 volumes of 96 per cent ethanol and some sodium acetate The solution was centrifuged and the precipitate was washed twice with 96 per cent ethanol and dried in vacuo above P_2O_5

The capsular polysaccharides isolated after treatment with liver extract were compared with the same polysaccharides oxidized by means of sodium periodate and the unoxidized capsular polysaccharides Antisera from rabbits against the three strains mentioned above were the same as those used in the previous paper The gel precipitation method used has been described earlier (4)

RESULTS

The type 1(A) capsular polysaccharide treated with liver extract seemed to be identical with capsular polysaccharide oxidized with sodium periodate when they both were used as antigens in a gel precipitation against homologous anti *Klebsiella pneumoniae* type 1(A) serum as shown in Fig 1 The liver extract seemed to attack the polysaccharide in the same way as periodate oxidation

The same result was obtained when the capsular polysaccharide from *Klebsiella* type 3(C) was treated with the liver extract This can be seen from Fig 2 in spite of the fact that the capsular polysaccharide after treatment with liver extract still contains polysaccharide of the original specificity (see Fig 8)

In anti *Klebsiella ozaenae* type 4(D) serum the result of the gel precipitation was different The capsular polysaccharide after treatment with liver extract did not show any difference from the untreated polysaccharide, nor did the periodate oxidized capsular polysaccharide precipitate any antibody at all This observation can be seen from Fig 3 These results indicated that the liver extract contained an agent prob



Fig 1

Gel precipitation in anti *Klebsiella pneumoniae* type 1(A) serum As antigens 1) Homologous capsular polysaccharide 2) The same polysaccharide treated with liver extract 3) The same polysaccharide oxidized with periodate

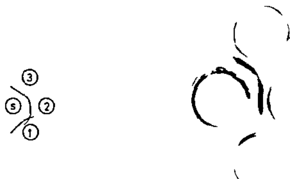


Fig 2

Gel precipitation in anti *Klebsiella* type 3(C) serum As antigens 1) Homologous capsular polysaccharide 2) The same polysaccharide treated with liver extract 3) The same polysaccharide oxidized with periodate



Fig 3

Gel precipitation in anti *Klebsiella ozaenae* type 4(D) serum As antigens 1) Homologous capsular polysaccharide 2) The same polysaccharide treated with liver extract 3) The same polysaccharide oxidized with periodate

ably an enzyme which attacked the capsular polysaccharides isolated from *Klebsiella pneumoniae* type 1(A) and from *Klebsiella* type 3(C) in the same way while the same agent had no influence on the capsular polysaccharide from *Klebsiella ozaenae* type 4(D) When the agent had effect on the polysaccharides it seemed to give the same results as oxidation with periodate Therefore it indicated some similarity in the oxidation products from the two polysaccharides whether the oxidation was due to periodate or to the agent present in the liver extract



Fig 4

Gel precipitation in anti *Klebsiella* type 3(C) serum As antigens 1) Homologous capsular polysaccharide 2) Homologous capsular polysaccharide oxidized with periodate 3) *Capsular polysaccharide from Klebsiella pneumoniae type 1(A) oxidized* with periodate 4) Capsular polysaccharide from *Klebsiella pneumoniae* type 1(A)

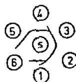


Fig 5

Gel precipitation in anti *Klebsiella pneumoniae* type 1(A) serum As [illegible]
1) Homologous capsular polysaccharide 2) Homologous capsular p[illegible]
oxidized with periodate 3) Capsular polysaccharide from *Klebsiella* [illegible]
oxidized with periodate 4) Capsular polysaccharide from *Klebsiell*[illegible]

From Fig 4 can be seen that both the oxidized homolo[illegible] saccharide and the oxidized capsular polysaccharide from [illegible] *pneumoniae* type 1(A) precipitated antibody from anti-*Kleb*[illegible] 3(C) serum The same results were obtained when anti[illegible] the type specific polysaccharide was removed beforehand

In anti-*Klebsiella pneumoniae* type 1(A) serum, oxidized [illegible] polysaccharide as well as oxidized capsular polysacc[illegible] *Klebsiella* type 3(C) precipitated antibody when used as [illegible]



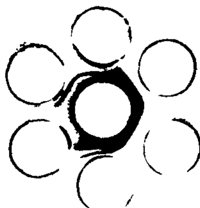


Fig 6

Gel precipitation Anti *Klebsiella pneumoniae* type 1(A) serum in the central well As antigens 1) Homologous capsular polysaccharide 2) The same polysaccharide oxidized with periodate 3) The same polysaccharide after treatment with liver extract 4) Capsular polysaccharide from *Klebsiella* type 3(C) oxidized with periodate 5) Capsular polysaccharide from *Klebsiella* type 3(C) treated with liver extract 6) Capsular polysaccharide from *Klebsiella* type 3(C)

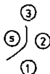


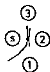


Fig 7

Gel precipitation in anti *Klebsiella pneumoniae* type 1(A) serum As antigens 1) Homologous capsular polysaccharide 2) Capsular polysaccharide from *Klebsiella* type 3(C) 3) Capsular polysaccharide from *Klebsiella ozaenae* type 4(D) All after treatment with liver extract

gel precipitation can be seen in Fig 5 The same results from gel precipitation were obtained whether the polysaccharides were oxidized by periodate or by the agent present in the liver extract The similarity in the way of oxidation can be seen from Fig 6

One reason why capsular polysaccharide isolated from *Klebsiella ozaenae* type 4(D) treated with periodate showed no precipitation in



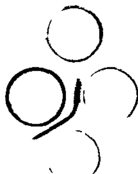


Fig 8
Gel precipitation in anti *Klebsiella* type 3(C) serum with the same antigens as used in Fig 7

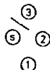


Fig 9
Gel precipitation in anti *Klebsiella ozaenae* type 4(D) serum with the same antigens as used in Figs 7 and 8

anti type 4(D) serum could be that this antiserum contained no measurable quantity of antibody against the oxidized polysaccharide The polysaccharide from type 4(D), treated with liver extract did not precipitate antibody in anti type 1(A) and type 3(C) sera, which can be seen in Figs 7 and 8 The liver extract seemed to have no influence at all on the type specific capsular polysaccharide isolated from *Klebsiella* type 4(D) The precipitation line seen in Fig 9 is the same line as in Fig 3 which showed identity with the capsular polysaccharide In all the precipitations described above, no difference could be seen whether the polysaccharides were treated with liver extract for 4 or 16 hr

DISCUSSION

In the previous paper it was indicated that oxidation could be one way the rabbit used for decomposing capsular antigens. This paper confirms the presence of an agent, probably an enzyme, in the liver of the rabbit which is responsible for the oxidation of the capsular antigens. However, this agent does not seem to have the same effect on all polysaccharides. The addition of liver extract to capsular polysaccharide isolated from *Klebsiella ozaenae* type 4(D) seemed to have no effect at all. No difference could be seen by gel precipitation when the capsular polysaccharide was used as antigen, before and after treatment with liver extract. This could be due to several reasons.

There has never been demonstrated antibody which reacted only with oxidized capsular polysaccharide in rabbit antiserum against this type. By oxidation with periodate, the polysaccharide will be much degraded. Even after oxidation for fourteen days, the polysaccharide still used periodate. The consumption of periodate was then 2.56 mol *of periodate per mol anhydrosugar. This indicated an overoxidation* which often is the case when the polysaccharide contains uronic acid, particularly when this is present as non reducing end group or linked through the 2-position (2).

Little is known about the structure of the capsular polysaccharide from *Klebsiella ozaenae* type 4(D) (6). By oxidation with sodium periodate it showed to be quite different from the capsular polysaccharides isolated from *Klebsiella pneumoniae* type 1(A) and *Klebsiella* type 3(C) concerning the linkages.

The two other capsular polysaccharides mentioned above, showed both to be attacked by the agent in the liver extract from the rabbit. The polysaccharide from *Klebsiella pneumoniae* type 1(A) is linked 1,3 and the polysaccharide from *Klebsiella* type 3(C) is linked mostly 1,3, but also 1,4 as shown by earlier investigations (1,3). By periodate oxidation the type 1(A) polysaccharide will only be attacked at the end group, while type 3(C) polysaccharide also will be attacked where the linkage is 1,4. Since the agent seemed to attack the two polysaccharides in the same way, and in a way not to be distinguished from the periodate oxidation, it is most likely that the agent attacks the end groups.

It must be borne in mind that the liver used for the extract was removed from a rabbit which for a long period of time had been immunized with a living culture of *Klebsiella ozaenae* type 3(C). This could also be an explanation why the agent did not attack type 4(D) polysaccharide. The agent present in the liver from the rabbit could be an adaptive and not a constitutive enzyme, in spite of the fact that it reacted both with type 3(C) and type 1(A) polysaccharides.

Another interesting thing about these investigations is the fact that oxidized polysaccharide from *Klebsiella* type 3(C) precipitates antibody

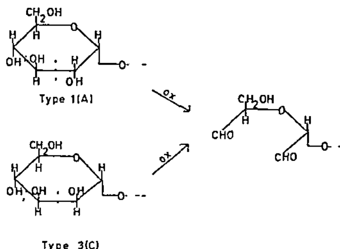


Fig 10

Oxidation of the non reducing end group in the capsular polysaccharides from *Klebsiella pneumoniae* type 1(A) and *Klebsiella* type 3(C)

from anti-*Klebsiella pneumoniae* type 1(A) serum and vice versa, whether the polysaccharides are oxidized by the liver extract or by sodium periodate

There is no cross-reaction between the capsular polysaccharides isolated from *Klebsiella pneumoniae* type 1 (A) and *Klebsiella* type 3(C) These two polysaccharides have been closely investigated (1,3) and it has been shown that they do not have any monosaccharide unit in common However, there are other facts which could explain the cross-reaction between the two oxidized polysaccharides concerned

The non reducing end group in the capsular polysaccharide from *Klebsiella pneumoniae* type 1(A) is glucose, proved by isolation of 2,3,4,6-tetra-0 methyl-glucose after methylation and hydrolysis The capsular polysaccharide from *Klebsiella* type 3(C) has mannose as non-reducing end group, proved by isolation of 2,3,4,6-tetra-0 methyl-mannose By oxidation, either with liver extract or with periodate, the same dialdehyde group will be formed from both the non reducing end groups as shown in Fig 10 The importance of chemical differences in the end group when the polysaccharides were used as antigens has been shown in the previous paper (5) as well as in previous papers by others (7)

The nature and mode of action of the oxidizing agent in the rabbit liver extract is considered to be outside the scope of the present study, and must be left for future studies

SUMMARY

The liver extract of a rabbit which had been immunized with *Klebsiella ozaenae* type 3(C) was found to contain an agent, probably an enzyme, which oxidized the capsular type specific polysaccharide

The same agent also oxidized the capsular type specific polysaccharide isolated from *Klebsiella pneumoniae* type 1(A), but had no effect on the capsular polysaccharide from *Klebsiella ozaenae* type 4(D)

Oxidized capsular polysaccharide from *Klebsiella pneumoniae* type 1(A) precipitated antibody from *Klebsiella type* 3(C) antiserum and oxidized capsular polysaccharide from *Klebsiella* type 3(C) precipitated antibody from anti *Klebsiella pneumoniae* type 1(A) serum in spite of the fact that there is no cross reaction between the two unoxidized polysaccharides The reason for this may be that oxidation—by liver extract or by periodate—may create identical end groups in both polysaccharides

REFERENCES

1 *Barker S A Brimacombe J S Eriksen J L & Stacey M* Capsular polysaccharide of *Klebsiella pneumoniae* type A (strain 1265) Nature (Lond) *197* 899 900 1963
2 *Edington R A Hirst E L & Percival Elizabeth E* The synthesis of methyl ethers of mannuronic and glucuronic acid and their reaction with periodate J chem Soc 2281 2288 1955
3 *Eriksen J* Immunochemical studies on some serological cross reactions in the *Klebsiella* group 10 Structure of the capsular polysaccharide of *Klebsiella* type 3(C) Acta path et microbiol scand nav *64* 347–361 1965
4 *Eriksen J* Immunochemical studies on some serological cross reactions in the *Klebsiella* group 12 Serological reactions with the acidic capsular polysaccharides from *Klebsiella* type 3(C) as antigens Acta path et microbiol scandinav *64* 522 526 1965
5 *Eriksen J* Immunochemical studies on some serological cross reactions in the *Klebsiella* group 14 Oxidized capsular polysaccharides as antigens Acta path et microbiol scandinav *65* 426 434 1965
6 *Joner P E* Immunochemical studies of *Klebsiella ozaenae* type D Acta path et microbiol scandinav 54 121 125 1962
7 *Kabat E A & Mayer M M* Experimental immunochemistry Charles C Thomas *Springfield Illinois* 1961
8 *Morton R K* Methods of extraction of enzymes from animal tissues In Methods in enzymology Colowick S P & Kaplan N O eds Academic Press Inc, New York *I* 25 1955

Acta path et microbiol scandinav 66 71 78 1966

Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt Oslo University Oslo
(Head Professor S Dick Henriksen M D)

IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS REACTIONS IN THE *KLEBSIELLA* GROUP

16 *Neutral Polysaccharide Antigens*

By

JORUNN ERIKSEN

Received 31 vii 65

Earlier work dealing with the chemical analysis of the capsular polysaccharide of *Klebsiella* type 3(C) indicated that the polysaccharides isolated from the four species *Klebsiella pneumoniae* (strain F 10.N Y), *Klebsiella ozaenae* (strain 3828 60), *Klebsiella rhinoscleromatis* (strain 92 04) and *Aerobacter* (*Klebsiella*) *aerogenes* (strain M A 73), all belonging to sero type 3(C) were identical (2) Serological investigations with the same polysaccharides used as antigens confirmed these results (7)

However, by the quantitative precipitin determinations in homologous and heterologous antisera a second antigen seemed to be present This antigen reacted only in homologous antiserum which meant that the capsular polysaccharide used as antigen must be a mixture of at least two antigens one of which reacting in homologous antiserum only Recent investigations (3) of the structure of the capsular polysaccharide of *Klebsiella* type 3(C) confirmed that the crude capsular polysaccharide was a mixture of two fractions one acidic and one neutral The acidic fraction was the type specific polysaccharide which has been examined in detail The purpose of this paper will be an investigation of the neutral fraction isolated from *Klebsiella* type 3(C) but also from other sero types of *Klebsiella*

MATERIAL AND METHODS

For purification of the crude polysaccharide isolated by cold water extraction cetyl pyridinium chloride was used (11) The acidic polysaccharide was precipitated as a complex with cetyl pyridinium chloride while the neutral polysaccharide remained
super
were
polys
washed three times with 96 per cent ethanol and dried in vacuo above P O_5

The neutral polysaccharide from the f llowing strains was isolated in this way

Klebsiella pneumoniae strain F 10 N Y
Klebsiella pneumoniae strain 1204

Klebsiella pneumoniae strain 270/60
Klebsiella o aenae strain 3828/60
Klebsiella o aenae strain 976
Klebsiella o aenae strain 054
Klebsiella rhinoscleromatis strain 92/04
Aerobacter (Klebsiella) aerogenes strain M A 73
They all belonged to sero type 3(C)

Neutral polysaccharides from other sero types were also isolated
Klebsiella pneumoniae type 1(A) strain 1265
Klebsiella pneumoniae type 2(B) strain F 24
Klebsiella o aenae type 4(D) strain 4461/62
Klebsiella o aenae type 5(E) strain 025

To get rid of the last trace of acidic polysaccharide gel filtration was used (5) The bed material was Sephadex G 75 coarse from Pharmacia Uppsala Sweden A glass column 500 mm long and 20 mm in diameter was packed in the recommended way (5) Water was used for equilibration packing of the column and for development Fractions of 5 ml were collected To identify the neutral polysaccharide the fractions were used as antigens in a gel precipitation against homologous antiserum

When the neutral polysaccharides had been localized by means of gel precipitation the fractions were combined and reprecipitated with three volumes of 96 per cent ethanol The precipitate was washed three times with 96 per cent ethanol centrifuged in the cold and dried in vacuo above P_2O_5

To compare the neutral polysaccharides before and after gel filtration the polysaccharides were hydrolysed and the monosaccharides were identified by paper chromatography The solvent developers and the spraying reagents were the same as used earlier (3)

Antisera from rabbits were produced as described earlier (4)

Gel precipitations were used as serological method (4)

RESULTS

The crude capsular polysaccharide was first separated into two fractions by cetyl pyridinium chloride Paper chromatography of the hydrolysates from the neutral fraction showed that this still contained traces of the acidic polysaccharide A content of galactose also seemed to dominate in each of the hydrolysates

When the neutral fraction was further purified by gel filtration the hydrolysates showed a different composition The uronic acid had disappeared which meant that the separation from the acidic fraction was successful The content of galactose in the hydrolysates from all strains had decreased which indicated that the neutral fractions also had been contaminated with a small quantity of galactan before gel filtration The monosaccharides present in the hydrolysates of the neutral fractions can be seen from Table I

The neutral polysaccharide fractions purified by gel filtration were used as antigens in gel precipitations They seemed to contain at least two antigens

The polysaccharide fractions from the eight strains belonging to sero type 3(C) which have been examined seemed all to contain an antigen in common This antigen reacted identically in anti type 3(C) sera from the different strains

TABLE 1

The Content of Monosaccharides in the Hydrolysates of the Neutral Fractions

Name	Type	Strain	Galactose	Glucose	Mannose
K pneumoniae	3(C)	F 10 NY	++	++	++
K pneumoniae	3(C)	1204	++	++	+
K pneumoniae	3(C)	270/60	++	++	+
K ozaenae	3(C)	3828 60	+++	++	++
K ozaenae	3(C)	076	+++	++	+
K ozaenae	3(C)	054	+++	++	++
K rhinoscleromatis	3(C)	92 04	+++	+	+++
K aerogenes	3(C)	M A 73	+	++++	
K pneumoniae	1(A)	1965	+++	+++	
K pneumoniae	2(B)	F 24	+++	+	+++
K ozaenae	4(D)	4461/62	+++	+	+
K ozaenae	5(E)	025	+	+++	+++

The second antigen, which was present in the neutral fraction, gave another reaction The antigen isolated from *Klebsiella pneumoniae* type 3(C) strain F 10 N Y seemed to be identical with those isolated from *Klebsiella pneumoniae* type 3(C) strain 1204 and strain 270 60, but showed no reaction with that isolated from *Klebsiella ozaenae* type 3(C) strain 3828 60 This can be seen in Fig 1, line a_2

On the other hand, when the neutral polysaccharide isolated from *Klebsiella ozaenae* type 3(C) strain 3828 60 was used as antigen, identity was obtained with the antigens isolated from *Klebsiella ozaenae* type 3(C) strain 054 and strain 076, but no reaction could be seen with the antigens from *Klebsiella pneumoniae* type 3(C) This is illustrated in Fig 2, line a_3

For some of the strains belonging to *Klebsiella type* 3(C) there seemed to be not only one but two antigens in common From Fig 3 it can be seen that *Klebsiella ozaenae* type 3(C) strain 3828 60 and *Klebsi-*

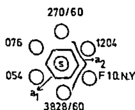


Fig 1

Anti *Klebsiella pneumoniae* type 3(C) strain F 10 N Y serum in the central well Neutral polysaccharide fractions as antigens in the peripheral wells The precipitation lines a_1 a_2 and a_3 in the different figures are identical

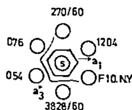


Fig 2

Anti *Klebsiella ozaenae* type 3(C) strain 3828 60 serum in the central well

Klebsiella pneumoniae strain 270 60
Klebsiella ozaenae strain 3828 60
Klebsiella ozaenae strain 076
Klebsiella ozaenae strain 0̲54
Klebsiella rhinoscleromatis strain 92 04
Aerobacter (Klebsiella) aerogenes strain M A 73
They all belonged to sero type 3(C)

sero types were also isolated
strain 1265
strain F 24
in 4461/62
n 025

To get rid of the last trace of acidic polysaccharide gel filtration was used (5) The bed material was Sephadex G 75, coarse from Pharmacia Uppsala Sweden A

ethanol The precipitate was washed three times with 96 per cent ethanol, centrifuged in the cold and dried in vacuo above P_2O_5

To compare the neutral polysaccharides before and after gel filtration the polysaccharides were hydrolysed and the monosaccharides were identified by paper chromatography The solvent developers and the spraying reagents were the same as used earlier (3)

Antisera from rabbits were produced as described earlier (4)

Gel precipitations were used as serological method (4)

RESULTS

The crude capsular polysaccharide was first separated into two fractions by cetyl pyridinium chloride Paper chromatography of the hydrolysates from the neutral fraction showed that this still contained traces of the acidic polysaccharide A content of galactose also seemed to dominate in each of the hydrolysates

When the *neutral fraction was further purified by gel filtration*, the hydrolysates showed a different composition The uronic acid had disappeared which meant that the separation from the acidic fraction was successful The content of galactose in the hydrolysates from all strains had decreased which indicated that the neutral fractions *also had been* contaminated with a small quantity of galactan before gel filtration The monosaccharides present in the hydrolysates of the neutral fractions can be seen from Table 1

The neutral polysaccharide fractions, purified by gel filtration, were used as antigens in gel precipitations They seemed to contain at least two antigens

The polysaccharide fractions from the eight strains belonging to sero type 3(C) which have been examined, seemed all to contain an antigen in common This antigen reacted identically in anti type 3(C) sera from the different strains

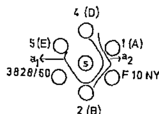


Fig 7
Anti *Klebsiella pneumoniae* type 2(B) serum in the central well

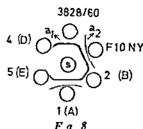


Fig 8
Anti *Klebsiella pneumoniae* type 3(C) strain F 10 NY serum in the central well

antigen shown in Figs 1 and 2 line a_1. With precipitation in anti *Klebsiella pneumoniae* type 2 (B) serum the same results were obtained. From Fig 7 there seemed to be one antigen in common for *Klebsiella pneumoniae* type 1(A) 2(B) 3(C) and *Klebsiella ozaenae* type 3(C). This antigen is probably also present in the polysaccharide fractions from *Klebsiella ozaenae* type 4(D) and 5(E) since the precipitation line is turning against the wells containing those antigens.

Just the same precipitation lines can be seen from Fig 8 where the gel precipitation was carried out against anti *Klebsiella pneumoniae* type 3(C) serum. There again a common antigen seemed to be present for *Klebsiella ozaenae* type 3(C), *Klebsiella pneumoniae* type 1(A), 2(B) and 3(C) and most probably also for *Klebsiella ozaenae* type 4(D) and 5(E).

From the Figs 6, 7 and 8 another antigen which is identical for *Klebsiella pneumoniae* type 1(A) and type 3(C) can be seen. This antigen is present not only in gel precipitations with anti *Klebsiella* type 1(A) and type 3(C) sera but also in anti *Klebsiella pneumoniae* type 2(B) serum. *Klebsiella pneumoniae* type 2(B) must in fact contain this antigen but the isolated neutral polysaccharide fraction from *Klebsiella pneumoniae* type 2(B) did not contain enough to give a precipitation line.

Klebsiella ozaenae type 3(C), type 4(D) and type 5(E) also seemed to contain an antigen in common. This is demonstrated in Fig 9 line

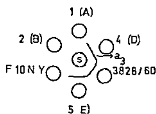


Fig 9
Anti *Klebsiella ozaenae* type 5(E) serum in the central well

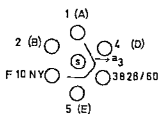


Fig 10
Anti *Klebsiella ozaenae* type 4(D) serum in the central well

a_3, which is a gel precipitation against *Klebsiella ozaenae* type 5(E) antiserum Just the same picture was obtained when the gel precipitation was carried out with anti-*Klebsiella ozaenae* type 4(D) serum as seen in Fig 10, line a_3

DISCUSSION

By further purification of the polysaccharide obtained by cold water extraction of *Klebsiella*, a neutral polysaccharide was isolated At first this fraction seemed to contain a single antigen which reacted in homologous antiserum only But after additional purifications, the neutral polysaccharide was found to contain more antigens These polysaccharide antigens must be easily soluble, since they can be demonstrated by extraction of the bacteria by cold water However, by cold water extraction they were only obtained in small quantities This was the reason why a closer examination of the polysaccharides was difficult to carry out It is an open question if these antigens can be obtained in a bigger yield when other methods are used for the extraction

In 1949 *Kauffmann* described the serological reactions of the *Klebsiella* group (8) as dependent on three factors

1 The capsular antigen = K-antigen
2 The somatic smooth antigen = O antigen, and
3 The somatic rough antigen = R-antigen

Thus the neutral polysaccharide fraction could be constituents of the O or R antigens, both of which would be expected to contain polysaccharide components

In Kauffmann's work the O-antigens were divided into three groups, which were composed of subgroups

In 1954, further examinations of the O-antigens were published by *Ørskov* (12) The three O-antigen groups were extended to five, with subgroups Later, the O antigens were described as consisting of nine different groups by *Durlakowa* (1), to be extended to ten groups in 1962 by *Maresz-Babczyszyn* (9)

According to *Kauffmann* the O-antigens of *Klebsiella pneumoniae* type 1(A) and type 3(C) and some strains of type 2(B) belonged to O-group 1, whereas other strains of type 2(B) as well as *Klebsiella rhinoscleromatis* type 3(C) and *Klebsiella ozaenae* types 4(D), 5(E), and 6(F) were assigned to O group 2

In the neutral polysaccharide fractions isolated from *Klebsiella pneumoniae* type 1(A), type 2(B) and type 3(C), an antigen was observed which gave reaction of identity, and which was not detected in the other strains This observation is compatible with those of *Kauffmann*, and there is a possibility that this antigen could be related to the O-antigen (Figs 6, 7 and 8, line a_2)

This antigen was also present in the other strains of *Klebsiella pneumoniae* type 3(C) (Fig 1, line a_2)

The same might be true of the antigens contained in *Klebsiella ozaenae* type 3(C), 4(D) and 5(E) which likewise showed reactions of identity in gel precipitations, in view of the fact that the types 4(D), 5(E) and 6(F) were placed in the same subdivision of O group 2 by *Kauffmann* (Figs 2, 9 and 10, line a_3) This could be a species specific antigen, which is in agreement with results reported earlier (6)

From the gel precipitations carried out with the neutral polysaccharides as antigens, there seemed to be one antigen in common to all the *Klebsiella* strains investigated (Figs 1, 2, 6, 7 and 8, line a_1) This observation could be in agreement with results reported earlier by *Pickett & Cabelli* (10) where another kind of antigenic layer, the "Sm" antigen was described This antigenic layer was distinct from the capsular and O-antigens, and was believed to be situated beneath the capsular antigen The nature of this layer was not reported

The available evidence with respect to a third antigen, which appeared to be present in some of the neutral fractions (Figs 3 and 4) is insufficient for a discussion of its nature

It is obvious that more than one antigen of carbohydrate origin distinct from the capsular polysaccharide is present in the *Klebsiella* group Nothing is known about the structure of the neutral polysaccharides, mostly due to lack of material, but they must have quite similar composition without being identical

SUMMARY

A neutral polysaccharide fraction has been isolated from eight strains of *Klebsiella* type 3(C) and from four strains of other sero types The neutral polysaccharide was different from the type specific polysaccharide concerning antigenic properties

1 One antigen seemed to be common for all strains investigated
2 A species specific antigen was demonstrated for *Klebsiella ozaenae* type 3(C), type 4(D) and type 5(E)
3 *Klebsiella pneumoniae* type 1(A), type 2(B) and type 3(C) seemed to share a second identical antigen
4 A third antigen appeared to be present in some of the neutral fractions

REFERENCES

1 *Durlakowa I Przondo Hessek A & Maresz Babczyszyn J* O antigens of *Klebsiella* types K 63–K 72 Arch Immunol Ter dosw *8* 645 654 1960
2 *Eriksen J & Henriksen S D* Immunochemical studies on some serological cross reactions in the *Klebsiella* group 6 Chemical analysis of the capsular polysaccharide of *Klebsiella* type 3(C) and of two cross reacting strains Acta path et microbiol scandinav *54* 387 390 1962
3 *Eriksen J* Immunochemical studies on some serological cross reactions in the *Klebsiella* group 10 Structure of the capsular polysaccharide of *Klebsiella* type 3(C) Acta path et microbiol scandinav *64* 347 361 1965

4 *Eriksen J* Immunochemical studies on some serological cross reactions in the *Klebsiella* group 12 Serological reactions with the acidic capsular polysaccharides from *Klebsiella* type 3(C) as antigens Acta path et microbiol scandinav *64* 522 526 1965
5 *Flodin P* Dextran gels and their applications in gel filtration Pharmacia Uppsala Sweden Meijels Bokindustri third edt Halmstad 1963
6 *Henriksen S D* Sero types of a collection of mucoid Gram negative rods Acta path et microbiol scandinav 26 424–435, 1949
7 *Henriksen S D & Eriksen J* Immunochemical studies on some serological cross reactions in the *Klebsiella* group 7 Serological reactions of some strains of type 3(C) and some cross reacting strains Acta path et microbiol scandinav 54 391–397, 1962
8 *Kauffmann F* On the serology of the *Klebsiella* group Acta path et microbiol scandinav 26 381 406, 1949
9 *Maresz Babczyszyn J* New antigenic types of *Klebsiella* Bacilli Arch Immunol Ter exp *10* 589 617 1962
10 *Pickett M J & Cabelli V J* The precipitating antigens of Friedlander s bacillus J gen Microbiol *9* 249 256 1953
11 *Scott J E* The reaction of long chain quaternary ammonium salts with acidic polysaccharides Chem & Ind 168–169 1955
12 *Orskov I* O antigens in the *Klebsiella* group Acta path et microbiol scandinav 34 145–156 1954

Acta path et microbiol scandinav 66, 79-92, 1966

The University Institute of General Pathology, Copenhagen, Denmark

STUDIES OF THE ANTIGENIC STRUCTURE OF MYCOBACTERIA

Report 2

By

K A Jensen, I Kiær and L Lundberg

Received 4 vi 65

In Report 1 (1) we demonstrated that the Schultz-Dale method could be employed for the antigenic analysis of mycobacteria We used three strains, *viz* , a human, an avian, and a fast-growing soprophyte (1500), which showed great differences in virulence, rate of growth, morphology of colonies, and biochemical characteristics We were able to demonstrate with great certainty that, besides a common antigen, these strains possessed a strain-specific antigen

In the present work we have extended these experiments to the mycobacteria listed in Table 1

From these very comprehensive experiments in which more closely related mycobacteria were included, we gained valuable experiences using the Schultz Dale technique

Firstly, it turned out that a few of the guinea pigs could not be sensitized or were so weakly sensitized that they could not be used in the experiments In order to ascertain whether the animals were suitable for these experiments, we tested the first piece of intestine with a heterologous antigen and afterwards with the homologous antigen If the intestine showed no reaction to the homologous antigen, we primarily tested the next piece of gut with the homologous antigen If a typical contraction of the intesttine could not be demonstrated, the animal was considered unsuitable

Secondly, it turned out that the pH value in the Ringer's solution which we were using had to be adjusted to 7 2

In all experiments mentioned below we have used the same batch of antigens for sensitizing the guinea-pigs as well as for the experiment itself Further, the reader is referred to Report 1, in which the technique employed is described

As the experiments were so comprehensive it has been considered more practicable for the sake of space to present the results in the form of simplified tables

Supported by grants from "King Christian X s Foundation" and "Miss P A Brandt s Foundation"

4 *Eriksen J* Immunochemical studies on some serolog cal cross reactions in the *Klebsiella* group 12 Serological reactions with the acidic capsular poly saccharides from *Klebsiella* type 3(C) as antigens Acta path et microbiol scandinav *64* 522 526 1965

5 *Flodin P* Dextran gels and their applications in gel filtration Pharmacia
Halmstad 1963

6
Gram negative rods Acta

7
dies on some serological cross reactions in the *Klebsiella* group 7 Serological reactions of some strains of type 3(C) and some cross reacting strains Acta path et micro biol scandinav *54* 391 397 196?

8 *Kauffmann F* On the serology of the *Klebsiella* group Acta path et microbiol scandinav *26* 381 406 1949

9 *Mares Babc ys yn J* New antigenic types of *Klebsiella* Bacilli Arch Immu nol Ter exp *10* 589 617 1962

10 *Pickett M J & Cabelli V J* The precipitating antigens of Friedlander s bacil lus J gen Microbiol *9* 249 256 1953

11 *Scott J E* The reaction of long chain quaternary ammonium salts with acidic polysaccharides Chem & Ind 168 169 1955

12 *Orskov I* O antigens in the *Klebsiella* group Acta path et microbiol scandinav *34* 145 156 1954

TABLE 1 (contd.)

Avian like strains		
No 31	Me 6484	G Meissner Tuberkulose Forschungsinstitut Borstel
No 58	Me 4846	G Meissner Tuberkulose Forschungsinstitut Borstel
No 60	Me 64	G Meissner Tuberkulose Forschungsinstitut Borstel
No 69	Me 2360	G Meissner Tuberkulose Forschungsinstitut Borstel
No 70	Me 914	G Meissner Tuberkulose Forschungsinstitut Borstel
M balnei strains		
No 32	Bal 7	Cultivated from skin affection Gothenburg Sweden
No 33	Bal 10	Cultivated from skin affection Gothenburg Sweden
No 34	Bal 14	Cultivated from the cemented walls of a swimming pool Örebro Sweden
No 35	Bal 16	Cultivated from skin affection Örebro Sweden
M kansasii strains		
No 36	Row	Selkon and Mitchison
No 37	Sv 1218	Cultivated from pulm nary resection Institut de Recherches de la Tuberculose Prague
No 38	687a	Sula, Czechoslovakia
No 39	499a	Sula, Czechoslovakia
No 40	735	Selkon and Mitchison
No 41	Me 1713	G Meissner Tuberkulose Forschungsinstitut Borstel
No 42	Me 6254	G Meissner Tuberkulose Forschungsinstitut Borstel
No 43	Me 1804	G Meissner Tuberkulose Forschungsinstitut Borstel
No 44	Me 11395	G Meissner Tuberkulose Forschungsinstitut Borstel
No 45	Me 1273	G Meissner Tuberkulose Forschungsinstitut Borstel
No 46	Me 10264	G Meissner Tuberkulose Forschungsinstitut Borstel
No 47	Me 11493	G Meissner Tuberkulose Forschungsinstitut Borstel
No 48	Me 7007	G Meissner Tuberkulose Forschungsinstitut Borstel
No 49	Me 2855	G Meissner Tuberkulose Forschungsinstitut Borstel
No 50	Me 3528	G Meissner Tuberkulose Forschungsinstitut Borstel
No 53	5824	Cultivated from a case of pulmonary ttuberculosis nine times within two and a half year University Institute of General Pathology Copenhagen
No 64	Me 4651	G Meissner Tuberkulose Forschungsinstitut Borstel
M battey strains		
No 68	4004 60	Cultivated from man
No 72	19580 60	Cultivated from pig Boyaes Australia
No 75	SSC 209	Cultivated from stomach lavage Statens Seruminstitut Copenhagen
No 77	19580 60	Cultivated from pig Boyaes Australia
No 78	11601/60	Cultivated from pig Boyaes Australia.
No 66	SN 285	Niacin positive fast growing R Bönicke Tuberkulose Forschungsinstitut Borstel
No 67	SN 281	Niacin negative fast growing R Bönicke Tuberkulose Forschungsinstitut Borstel
No 71	SN 286	Niacin positive fast growing, R. Bonicke Tuberkulose Forschungsinstitut. Borstel
No 73	SN 282	Niacin negative fast growing R Bonicke Tuberkulose Forschungsinstitut Borstel
No 74	SN 284	Niacin positive fast growing R Bonicke Tuberkulose Forschungsinstitut Borstel
No 79	SN 288	Niacin negative fast growing, R. Bonicke Tuberkulose Forschungsinstitut Borstel
No 51	Ty 505	Cultivated three three times from sputum F Dosch Hygienisch-Bakteriologische Untersuchungsanstalt Wien
No 52	1500	Cultivated two times from a case of pulmonary tuberculosis University Institute of General Pathology Copenhagen

Studies on the Antigenic Structure of Human and Bovine Strains – Group I

TABLE 2

Antigenic Structure of Human Bovine, and BCG strains – Group I

	1 H	2 H	3 H	9 H	17 BCG	16 BCG	20 B
1 H	4/4 +	2/2 +	2/2 +	2/2 +	2/2 +	2/2 +	2/2 +
2 H	2/2 +	2/2 +	1/1 +				
3 H	3 3 +	1/1 +	2/2 +	1/1 +	1/1 +	1/1 +	2/2 +
9 H	3/3 +	1/1 +	2/2 +	2/2 +	1/1 +	2/2 +	2/2 +
4 H	2/2 +	1/1 +					
5 H	1/1 +	1/1 +				1/1 +	
6 H	1/1 +	1/1 +					
7 H	1/1 +	1/1 +					
8 H	1/1 +	1/1 +					
10 H	1/1 +						
11 H	1/1 +						
12 H	1/1 +						
13 H	1/1 +	1/1 +					
14 H	1/1 +						
15 H	1/1 +	2/2 +					
17 BCG	3/4 +	1/2 +	1/1 +	1/2 +	2/2 +	2/2 +	2/2 +
16 BCG	1/2 +	1/1 o	2/2 +	2/2 o	1/1 +	2/2 +	1/2 +
20 B	2/2 +		2/2 +	1/2 +	2/2 +	2/2 +	2/2 +
18 BCG		1/1 +					
19 BCG	3/4 +	1/1 +	1/1 +				
21 B	1/1 +	1/1 o					
23 B		1/1 +					

24(25) A	11/11 o	6/6 o
31(58–60) Al	5/5 o	
32–33 34 35 Ba	28/28 o	18/22 o – 4/22 +
36–37 38 39 (40–41 42 43 44–50) K	30/30 o	8/11 o 3/11 +
53 K	10/10 o	4/5 o – 1/5 +
51 Ts	4/4 o	1/1 o
52 F	8/8 o	6/6 o

Explanations of Tables 2–6

The antigens used are recorded in the first column The strains showns in parentheses were employed in one test only The strains used to sensitize the experimental animals are shown across the top of the tables + means that the strain in question renders the gut refractory to the homologous antigen *i e* there is a strong probability that it contains the same specific antigen as the strain employed for sensitizing o means that the strain concerned does not make the gut refractory to the homologous antigen *i e*, it does not contain the specific antigen *Fractions* The numerator indicates the number of + or o reactions while the denominator indicates the number of experimental animals employed in the tests

H = human strains B = eugonic bovine A = avian Al = avian like
Ba = balnei K = kansasii Ts = 505 F = fast growing strain

From Table 2 it appears that the antigens from all the human strains can render the intestines sensitized with human or bovine antigens, refractory to the homologous antigens In other words, with the technique employed, the bovine strains do not possess an antigen which the

human strains lack If, however, we consider the cross experiment, it appears that in 10 out of 32 experiments the bovine antigens could not render the intestines, sensitized with human antigens, refractory to human antigens

The reason for this may be that the production of the group-specific antigen by the bovine strains is rather weakly defined, and therefore in some of the animals was not able to render the intestine refractory to the possibly more well developed group antigen from the human type If this is the case, a simplified antigenic formula will be X–1 for the human group and X–(1) for the bovine group, X being the common antigen and 1 the group specific antigen

The reason may be, however, that the human group also possesses a weak *type-specific antigen* (1a), *and the formula will thus be* X–1 (1a) for the human type and X–1 for the bovine type This latter formula seems to be the most probable one, as it appears from Table 2 (the lower part) that the human as well as the bovine strains can be clearly differentiated from the other mycobacteria

By means of other methods we are investigating the presence of a possibly type-specific human antigen

The Antigenic structure of M balnei – *Group II*

TABLE 3

Antigenic Structure of M balnei – *Group II*

	32 Ba	33 Ba	34 Ba	35 Ba
32 Ba	2/2 +	3/3 +	2/2 +	4/4 +
33 Ba	2/2 +	3/3 +	2/2 +	4/4 +
34 Ba	2/2 +	2/3 +	2/2 +	4/4 +
35 Ba	2/2 +	3/3 +	2/2 +	4/4 +
1 2 3 9 H		23/25 o – 2/25 +		
16 17 20 B		21/21 o		
24 25 26 59 A		19/19 o		
31(58 60) Al		6/6 o		
36 37 38 39 40–41 42–43 K		26/35 o – 9/35* +		
53 K		8/10 o – 2/10 +		
51 Ty		10/10 o		
52 F		9/9 o		
55 S		4/4 o		
66 SN		2/2 o		

For explanation see Table 2

S = saprophyte SN = niacin positive saprophyte Italic type signifies that that particular antigen was most frequently employed

* 6 of the 9 tests were carried out on gut taken from the same guinea pig

From Table 3 we can conclude with certainty that the four strains examined are identical with regard to antigenic structure and can clearly be distinguished from the other mycobacteria We can thus set up the antigenic formula for this group as X–2 This formula has no doubt been simplified, because—as will be seen from the table—in 11

TABLE 4

Antigenic Structure of M kansasii – Group III

	36 K	37 K	38 K	39 K	40 K	41 K	42 K	43 K	49 K	53 K
36 K	4/4 +	4/4 +	2/2 +	2/2 +	1/1 +	1/1 +	1/1 +	2/2 +	1/1 +	5/5 +
37 K	4/4 +	4/4 +	2/2 +	1/2 +	1/1 +		1/1 +	1/1 +	1/1 +	3/3 +
38 K	2/2 +	2/2 +	2/2 +	2/2 +	1/1 +	1/1 +	2/2 +	2/2 +	1/1 +	2/2 +
39 K	2/2 +	2/2 +	2/2 +	2/2 +	1/1 +	1/1 +	2/2 +	2/2 +	1/1 +	1/1 +
40 K	2/2 +		2/2 +	1/2 +	1/1 +	1/1 +	2/2 +		1/1 +	2/2 +
41 K	2/2 +	2/2 +	2/2 +	2/2 +	1/1 +	1/1 +	2/2 +	2/2 +	1/1 +	2/2 +
42 K	2/2 +	2/2 +	2/2 +	2/2 +	1/1 +	1/1 +	2/2 +	2/2 +	1/1 +	2/2 +
43 K	2/2 +	2/2 +	2/2 +	2/2 +	1/1 +	1/1 +	2/2 +	2/2 +	1/1 +	2/2 +
49 K	1/1 o	2/2 +	2/2 +	1/2 +	1/1 +	1/1 +	1/2 +	2/2 o	1/1 +	1/1 +
53 K	1/4 +	2/2 o	1/2 +	2/2 o	1/1 +	1/1 o	2/2 o	2/2 o	1/1 +	5/5 +
44 K	2/2 +	2/2 +	1/2 +	1/2 +	2/2 +		1/2 +	2/2 o	1/1 +	
45 K	1/1 +		1/1 +	1/1 o	1/1 +	1/1 +	1/1 +		1/1 +	
46 K	1/1 +	2/2 +	1/1 +				2/2 +		1/1 +	
47 K			1/1 +	1/1 +			2/2 +	2/2 +	1/1 +	
48 K		1/1 +	1/1 +	1/2 +	1/1 +	1/1 +	2/2 +	2/2 +	1/1 +	
50 K	1/2 +	2/2 +	1/2 +	2/2 o	1/1 o	1/1 o	2/2 o	2/2 o	1/1 +	
1 2 9 H					36/39 o – 3/39 +					
16–17 20 B					18/18 o					
24–25 26 27 63 A					26/27 o – 1/27 +					
31 56 Al					17/17 o					
32 33–34–35 Ba					28/33 o – 5/33 +					8/12 +
51 Ty					16/16 o					
52 F					15/15 o					
54 55–56 S					5/5 o					

For explanation see Tables 2 and 3

out of 45 experiments the *M. kansasii* antigens render the intestines, sensitized with *M. balnei* antigens refractory to the *M. balnei* antigens, and in 2 out of 25 experiments the human antigens render the intestines refractory to *M. balnei* antigens.

This result may indicate the presence of a weak antigen common to groups I, II, and III, which will be mentioned later, apart from the common antigen X.

The Antigenic Structure of M. kansasii – *Group III*

From Table 4 it appears that all the strains examined except four seem to be incorporated in a group which deviates from the other mycobacteria. The four deviating strains (44, 49, 50, and particularly 53) will be mentioned later. From this table it also appears that some antigenic overlapping exists between the groups I, II, and III as previously mentioned.

For the present we can set up the simplified antigenic formula as X–3.

The Antigenic Structure of the Avian Strains (Group IV) and the Avian Like Strains (Group IV–o)

TABLE 5

Antigenic Structure of the Avian Strains (Group IV) and the Avian like Strains (Group IV–o)

	24 A	25 A	26 A	31 Al	58 Al
24 A	9/9 +	2/2 +	2/2 +	2/2 +	1/1 +
25 A	7/7 +	2/2 +	2/2 +	2/2 +	1/1 +
26 A	7/7 +	2/2 +	2/2 +	2/2 +	1/1 +
27 A	3/3 +	2/2 +	1/1 +	1/2 +	
28 A	3/3 +	2/2 +	1/1 +	1/1 +	
29 A	3/3 +	2/2 +	1/1 +	1/1 +	
30 A	3/3 +	2/2 +		1/1 +	
57 A	5/5 +		1/1 o		
59 A	3/3 +	1/1 +	2/2 +		1/1 +
61 A	4/4 +				
62 A	2/2 +				1/1 +
65 A	5/5 +				1/1 +
63 A	1/7 +		1/1 +		1/1 o
31 Al	7/7 o	2/2 o	2/2 o	2/2 +	1/1 +
58 Al	4/4 o	1/1 o	1/1 o	2/2 +	1/1 +
60 Al	6/6 o	1/1 o	1/1 o	1/1 +	1/1 +
70 Al	2/2 o				
69 Al	2/2 o				
1 (2 3 9) H		18/18 o		3/4 o	1/4 +
17 B		8/8 o			
32(?) 33 34 35 Ba		22/22 o		4/4 o	
36 37–38 39 40–41 42 43 K		47/47 o		14/14 o	
53 K ?		10/10 o		3/3 o	
51 Ty		9/9 o		2/2 o	
52 F		8/8 o		2/2 o	
68 72 75(?) Bat		5/5 o			

For explanation see Tables 2 and 3. Bat = *M. Battey*

From Table 5 it appears that the antigens from all the avian strains with the exception of strain 63A are able to render the intestines sensitized with avian and avian like antigens refractory to the avian and avian like antigens. With the technique employed this shows that the avian like strains do not possess a type specific antigen which the avian strains lack. In the cross experiment the avian like are however unable to render the intestines sensitized with avian antigens refractory the the avian antigens. Tentatively we can therefore set up the antigenic formula for this group as follows: avian strains X–4 4a and avian like strains X–4.

Strain 63 A will be mentioned later.

It is of interest to note that the three strains of *M. battey* clearly deviate from the avian strains with regard to antigenic structure.

The Antigenic Structure of 51Ty (Group V)

TABLE 6

Antigenic Structure of 51 Ty (Group V) and 52 F (Group VI)

	51 Ty	52 F
51 Ty	3/3 +	1/1 o
H	4/4 o	6/6 o
B	2/2 o	6/6 o
A	15/15 o	2/2 o
Al	7/7 o	1/1 o
Ba	5/5 o	8/8 o
K	21/21 o	4/4 o
53 K	3/3 o	2/2 o
52 F	2/2 o	2/2 +

For explanation see Tables 2 and 3

With regard to the antigenic formula this single strain deviates from all other mycobacteria examined (Table 6).

This antigenic formula can thus be set up as X 5.

The Antigenic Structure of 52F (Group VI)

This strain also deviates from the other mycobacteria. The antigenic formula is X 6 (Table 6).

Our investigations of *M. battey* are far from complete as we have had some difficulties in cultivating these strains on Sauton's medium. Our preliminary investigations seem however to indicate that these strains belong to a special group as far as their antigens are concerned. At any rate apart from the common antigen X they do not possess the group specific antigens 3 and 4.

Investigations of the Avian Strain 63A, which Deviates from the Other Avian Strains

In order to examine this strain in cross immunization experiments, we sensitized two guinea pigs with 63 A and two with our standard avian strain 24 One guinea pig sensitized with 63A proved unsuitable, as the intestines did not react to any antigen The result of this experiment will be seen from Table 7

TABLE 7

Antigenic Studies on the Deviating Avian Strain 63 A

	24 A	24 A	63 A
24 A			4 – 0
63 A	1 3	0 3	
25 A	4 0	3 0	
26 A	4 – 0	3 – 0	
27 A	4 0	4 0	4 0
28 A	4 0		
29 A	4 – 0	4 0	
30 A	4 – 0	3 0	
57 A	4 0	3 0	
61 A	4 – 0	3 – 0	
62 A	4 – 0		4 0
65 A	4 – 0	3 1	
31 Al	2 4	1 2	4 0
58 Al	4 3	1 – 2	4 0
60 Al	4 3	2 – 2	4 0
69 Al	3 4	1 – 3	4 0
70 Al	2 4	1 – 3	4 – 1
1 H	0 4	0 – 4	0 3
17 B	0 4	0 4	0 4
35 Ba	2 4	0 3	
34 Ba	1 4		
72 Bat	2 4		4 0
75 Bat	0 4	1 3	4 2
68 Bat	1 4	1 3	4 0
36 K			2 – 3
38 K			2 – 3

For explanation see Tables 2 and 3

The figures 0 to 4 indicate the strength of the intestinal contraction The first figure indicates the strength of the contraction after employment of the first antigen i e the antigen appearing in the first column The second figure indicates the strength of the reaction to the homologous antigen (see top of table) after the gut has become refractory to the first antigen employed

The experiment shows that strain 63A does not render the intestine, sensitized with 24A, refractory to 24A, whereas 24A and the other avian strains render the intestine sensitized with 63A, refractory to 63A

It is of interest to note that four out of the five avian like strains are

also able to render the intestine, sensitized with 63A, refractory to 63A and that two out of the three strains of *M. batteyi* can render the intestine refractory to 63A.

This shows that the batch of antigens prepared from 63A either does not possess the antigen 4 specific to the avian group at all, or that it is present only in small quantities.

We are now investigating whether this strain really does deviate from the other avian strains with regard to antigens, or whether the batch of antigens used in the experiment does not possess the group-specific antigen.

Studies on the Deviating Strains of M. kansasii

TABLE

Antigenic Studies on the Deviating

	37 K	37 K	38 K	38 K	40 K	40 K	42 K
36 K	4 - 0	4 - 0	4 - 0	4 - 0		4 - 0	4 - 0
37 K					4 - 0		
38 K	4 0	4 0				4 - 0	
39 K	4 0	4 0	4 - 0			4 - 0	
40 K			4 - 0	4 0			
41 K	4 - 0	4 0	4 - 0	4 0	4 - 0	4 - 0	4 - 0
42 K	4 - 0	4 0	4 - 0				
43 K	4 - 0	4 0	4 0				
44 K	4 - 0	4 0	4 0	4 0	4 - 0	4 - 0	
45 K							
46 K	4 - 0	4 0					
47 K							
48 K		4 - 0					
49 K	4 - 0	4 - 0	4 - 0	4 0			4 - 0
50 K	4 0	4 - 0	4 0	4 0	4 - 0	4 - 1	4 - 2
53 K	4 1	4 - 2	4 2	4 1	3 - 2	4 - 0	4 - 4
64 K	4 0	4 - 0	4 0				
1 H	4 1	4 1	4 4	4 1	4 2	4 4	4 - 1
17 B				3 4	4 - 4		4 - 4
24 A	3 3	3 4	3 4	4 4	4 3	3 4	4 4
63 A			3 3				
31 Al				4 - 4	1 4	4 4	4 - 4
35 Ba	3 1	4 4	4 4			4 4	
34 Ba			4 3	4 3	4 - 3	4 3	4 4
68 Bat	1 - 3	3 - 4					
75 Bat	3 3	3 4	4 - 4				
72 Bat	3 3	3 4	4 4	4 4	4 4	2 - 4	4 4
71 SN				1 4	1 - 4	0 - 4	0 - 4
73 SN				0 - 4	0 4	0 4	0 - 4
51 Ty				4 4	4 - 3	3 4	3 - 4
52 F				3 4	3 4	4 4	2 - 4

For explanation see Table 7

From Table 8 it will be seen that the supplementary cross examination of strains 44K and 49K indicates that these strains are identical with the other strains of *M kansasii*

Strain 50K shows a few results deviating from 40K, 42K, and 49K, but if we consider the results as a whole, it will be seen that there are 10+ o and only 3+ – + reactions, while the intestines sensitized with 50K show a reaction of + – o to all the tested strains of *M kansasii* This seems to indicate that the three strains 44K, 49K, and 50K deviate from the other strains of *M kansasii* only because they produce a weaker group antigen

ins of M kansasii

	53 K	53 K	50 K	50 K	49 K	49 K	44 K	44 K
)	4 0	4 0	4 0	4 0	4 0			4 0
				4 0				
	4 0	4 0		4 0	4 0	4 – 0		
		4 0		4 0	4 0			
		4 – 0		4 – 0			4 0	
)	4 – 0	4 – 0	4 0	4 0	4 0	4 – 0		4 0
	4 0	4 0		4 0	4 0			4 0
		4 – 0		4 0	4 0			
		4 0	4 – 0	4 0		4 0		
				4 0				
		4 0		4 0	4 0			
				4 0				
		4 0		4 0	4 0			
)		4 0	4 – 0	4 0			4 – 0	4 – 0
}	4 0	4 3			4 1	4 0	4 – 0	4 – 0
›			4 0	4 0	4 0	4 2	4 0	4 1
		4 0		4 0	4 – 0			
›	4 1	4 4	4 1	4 – 2	4 4	4 1	4 1	4 1
4			3 3					4 3
4	3 3	3 4	4 4		4 – 4	4 4	4 1	
	4 4		3 4			4 4	4 – 3	4 4
4	4 3					4 4	4 3	3 4
	3 1	3 4		4 1		4 1	4 1	
1	4 2	4 4	3 2	4 1	4 2			4 – 3
	3 1	4 4		4 4	3 – 4	3 2	4 2	
	3 1	2 4		4 3	3 4			
4	3 2	0 4	4 4	4 4	4 4		3 4	2 4
4			1 4					0 4
4			0 4	0 4				0 4
4	4 2		4 4					4 – 3
4	3 4		3 4					3 4

As far as strain 53K is concerned, it is seen to deviate considerably from the other strains in the cross experiment since it shows 9+ – + reactions and only 5+ – o reactions, while the intestines, sensitized with antigen 53, give reactions of + – o in all cases except 50K This result thus resembles the result obtained for the avian and the avian-like strains and the avian strain 63A This may, of course, be due to the possibility that 53K produces a weak group-specific antigen, but the reason may also be that it does not possess a type-specific antigen This last assumption is based on the circumstance that, in the experiments with guinea-pigs sensitized with 53K, we have with certainty been able to differentiate it from the other groups of mycobacteria

On the basis of the above mentioned results we have set up the following antigenic formulas for the various strains

M kansasii	X–3–3a
44K, 49,K, and 50K	X–3–(3a)
53K	X–3–?

Experiments with other batches of antigens from these four strains are at present being carried out

DISCUSSION

As in every other serological classification of bacteria the result of the method used in these experiments depends on the concentrations of the different antigens in the reagents used for immunization and the serological reaction The most reliable classification is obtained when only the dominant antigens are taken into consideration Conversely, such a classification will often only be suitable for group classification As is well-known, besides a very dominant common antigen which causes great difficulties in serological classification, the mycobacteria also possess numerous other antigens Several of these antigens are present in rather small quantities and can, in fact, vary considerably from one production of antigens to the other If therefore all antigens are used for a classification of mycobacteria, we may easily be misled and obtain wrong results The principle in the most reliable antigenic analysis is the cross-wise absorption, and the two strains can be stated to be identical only if the strains are able to absorb each other's antibodies completely By the word "state" we mean that the possibility of the two strains being identical is so great that this classification can be used in practice In cross absorption, rather large dilutions of the sera are used, and consequently some of the weak antibodies will disappear Therefore, this form of classification is not a complete antigenic analysis In the above experiments we have used the same principle as in the cross absorption since by rendering the intestines refractory to an antigen, we eliminate the antibody corresponding to this antigen Of course, this method also depends on the strength of the antigens in the

preparations employed Furthermore the concentration of a weak specific antigen may be below the limit where it is able to elicit a definite contraction of the intestine

The technique employed is thus suitable for a group classification of *mycobacteria* on the basis of the dominant antigens *alone* However also with this group classification certain irregularities have been observed For some groups we have been able to demonstrate with certainty the group specific antigen which differentiates them from the other groups—but a few strains did not fulfil the condition of a cross desensitization Among such groups we can mention group I (human bovine) group III (*M kansasii*) and groups IV IV–o and 63A (avian like strains)

TABLE 9

		Simplified antigenic formula	Hypothetical antigenic formula
Group I	Human	X 1	X 1 (1a) 1b
	Bovine	X (1)	X 1 ? (1b)
Group II	*M balnei*	X 2	X 2 1b
Group III	*M kansasii*	X 3	X 3 3a 1b
	50 K	X (3)	X 3–(3a) 1b
	53 K	X ((3))	X 3 1b
Group IV	*M avium*	X 4 4a	X–4 4a 4b
	63 A	X 4	X 4 (4a) 4b
Group IV o	Avian like	X 4	X 4– (4b)
Group V	51 Ty	X 5	X 5
Group VI	52 F	X 6	X 6

Simplified and Hypothetical Antigenic Formulas of the Different Strains
X indicates the common antigen The figures 1 6 indicate the group specific antigen Parentheses signify that the antigen is absent or very weak a = a possible type antigen b = an antigen that links Groups I II and III together in one related group and Groups IV and IV o in another related group

Therefore it cannot be desidered on the basis of the present investigations whether these irregularities arise because the group specific antigen in the deviating strains concerned is too weak or whether they may be due to a possible type specific antigen The first explanation however is most probable This problem is now being investigated On the basis of the present material the following preliminary antigenic scheme has been elaborated The simplified and certain formulas are shown in the first column the hypothetical ones in the second

The presence of a superior group antigen b cannot be ascertained from the tables presented in this paper

The proof of the presence of this antigen is based on the following three circumstances Firstly the strains within these superior groups

yield very strong primary reactions Secondly it may be difficult to render the intestines refractory Thirdly in quite a few cases the strains within these superior groups are able to render the intestine refractory to the homologous antigen

The correctness of this hypothetical antigenic formula is now being investigated by the employment of other serological methods for antigenic analysis

SUMMARY

By means of the Schultz Dale method it has been practicable with certainty to classify the examined mycobacteria into seven groups Group I comprises the human and bovine types Group II *M balnei* and Group III *M kansasii* Antigenically these three groups are closely related Group IV comprises the avian strains and Group IVa the avian like strains As far as Group V and VI are concerned only one strain has been demonstrated until now in each group These two strains have been cultivated several times from tuberculosis like diseases in man *A few deviating strains were demonstrated in Groups III and IV* and at present we are investigating whether these deviations are due to complete or partial lack of the group specific antigen in the preparation employed or whether it is a type specific antigen that is lacking

On the basis of our investigations an antigenic scheme has been set up as wille be seen from Table 9

After compilation of the present work guinea pigs were sensitized with three strains of *M battey* (Bat 68 75 and 77) and four strains of the fast growing SN two of which were niacin positive and two niacin negative (S66 67 73 and 74)

The three *M battey* strains were found to possess a group specific antigen which distinguishes them from the other seven groups besides the common antigen X The antigenic formula has thus been set up as X 7

Similarly the four SN strains were found to possess an antigen specific to this group The common antigen X is either not present at all or is very weak and thus with two exceptions the various mycobacteria employed do not elicit a primary reaction of guinea pig gut sensitized with SN The two exceptions referred to are Fa2 (1500)—a fast growing strain—and S55 These two strains do not however contain the antigen specific to the SN group Thus the formula for the SN strains is (X) Y8 in which Y is the common antigen for the SN strains and for Fa2 and S55 The formula for Fa2 has been set up as XY6 and for S55 as X Y)? The group specific antigen for this last strain has not been determined Undoubtedly there are numerous group specific antigens within the saprophytic mycobateria

Later a complete report of the investigations mentioned will be published

REFERENCE

1 *Jensen K A Kiær I & Lundberg L* Studies on the antigenic structure of *Mycobacteria* Report 1 Acta path microbiol scand na *61* 619 632 1964

Acta path et microbiol scandinav 66 93 104 1966

The Institute of Clinical Bacteriology University of Lund General Hospital Malmo

STUDIES ON NON-SPECIFIC ANTISTREPTOLYSIN O TITRE

I The Influence of Serum β Lipoproteins on the Non-Specific Antistreptolysin O Titre

By

STEN WINBLAD

Received 18 xi 65

False antistreptolysin titres caused by bacterial contamination on the sera were occasionally found by *Hewitt & Todd* as early as 1939 Other factors inhibiting streptolysin (SL) O were noticed by *Packalen* who found that sera from patients with jaundice, sera treated with acid or alkali, and pleural exudates showed abnormally elevated antistreptolysin O titres (AST), not to be explained as real antibody titres He concluded that this group of inhibitory substances consisted of lipids separable from the real antibody, e g by electrophoresis Further electrophoretic studies have demonstrated diverse peaks among the globulins caused by AST components (*Packalen, Hedberg, Scheiffarth et al*), and different peaks are obtained if the serum is fractionated by gel filtration (*Killander & Philipson*) Elimination of non-specific AST with bentonite or acetone-ester was tried by *Oker Blom, Nikkila & Kalaja* but without practical success *Cabau & Badin* found it useful to have albumin present in the reaction in order to diminish non-specific AST

On the hypothesis that the streptolysin inhibiting substance belongs to the lipoproteins, more specifically the *β*-lipoproteins, the logical course should be to eliminate such lipoproteins from the serum *Burstein & Samaille* as well *Bernfeld, Nisselbaum, Berkeley & Hanson* showed that sulphated polysaccharides, in the presence of metal ions, interact with the lipoproteins to form insoluble complexes Such an insoluble complex is formed by dextran sulphate in combination with $CaCl_2$, mainly by interaction with *β*-lipoproteins This method for depleting serum of lipoproteins appears to be more useful in practical work than ultracentrifugation which is also possible (*Legler et al*) *Hallen* used the method of *Burstein et al* to show that the highly elevated AST encountered in jaundice could by this means be reduced to low values as opposed to the elevated AST of rheumatic fever or tonsillitis

In a preliminary study we compared the albumin method and the dextran sulphate method, and we found the latter to be preferable

because of its striking influence on the AST of icteric sera and because of its simplicity. The dextran sulphate method was therefore chosen for the present investigation where we studied its capacity of reducing β lipoproteins in sera from healthy persons and patients with various diseases. Presumably, even healthy people have some amount of β lipoproteins capable of inhibiting SL O. The results of the electrophoretic studies mentioned above point in this direction. Rheumatoid arthritis is of special interest since it has been suspected that the relatively elevated AST that frequently occurs in this group represents a non-specific factor.

METHODS AND MATERIALS

Antistreptolysin reaction.—Streptolysin from a broth culture of the S 84 strain was used. The broth was prepared from ox heart with 2 per cent peptone, 0.2 per cent glucose, 0.2 per cent $NaHCO_3$ and 0.1 per cent Na_2HPO_4, pH adjusted to 8.0. The streptolysin-containing broth was centrifuged and filtered through a Seitz filter. The amount of streptolysin units was determined by titration against standard antistreptolysin serum after reduction with a 0.4 per cent sodium pyrosulphite solution. The standard antistreptolysin globulin was obtained from the Danish State Serum Institute of Copenhagen. A suitable amount of streptolysin broth containing 1 unit/ml was chosen. The patient's serum was diluted with normal saline in small tubes to a total volume of 1 ml per tube and was inactivated. 0.5 ml of streptolysin was added to each tube. After incubation of this mixture in a 37° C water bath for 15 minutes 0.5 ml of a 5 per cent suspension of sheep erythrocytes was added. The mixture was then incubated at 37° C for 30 minutes and allowed to stand for 1 hour or overnight at 4° C before the degree of haemolysis was determined colorimetrically. The amount of antistreptolysin units present was calculated from the number of combined streptolysin units.

β lipoprotein precipitation.—The β lipoproteins were precipitated by the method of Burstein & Samaille (1958) in which 0.02 ml of 10 per cent dextran sulphate and 0.1 ml of 1M calcium chloride solution are added to 1 ml of serum. One hour later the mixture was centrifuged for 10 minutes at 1500 G. The clear supernatant was decanted and AST was determined.

Fresh sera were used. The AST values of untreated and of β lipoprotein depleted serum were determined at the same time.

Origin of the sera.—146 sera were obtained from blood donors taken to represent healthy adult individuals. 194 sera were from patients with rheumatoid arthritis who were all positive with regard to the rheumatoid factor. 124 sera with AST $\geq$ 300 units were from patients with acute tonsillitis, acute nephritis, or rheumatic fever, i.e. patients supposed to be infected by β haemolytic streptococci and to have an elevated AST due to "real" antibody. Added to this were 67 sera from patients with non classified but non icteric diseases, 20 sera from patients with hypercholesterolaemia having cholesterol levels in serum of 400 mgs per cent or more, and finally 180 samples from 91 patients with jaundice from various causes recorded below.

RESULTS

The Decrease of AST in Different Sera. Depletion of β Lipoproteins by Dextran Sulphate

Blood donors. The 146 blood donors had AST values ranging from very low titres up to 600 units (Fig. 1). After depletion of the β lipoproteins the AST values fell about one dilution step and were decreased to 50 per cent of their previous numeric value. Table 1 shows the median AST values in sera with and without β lipoproteins from blood

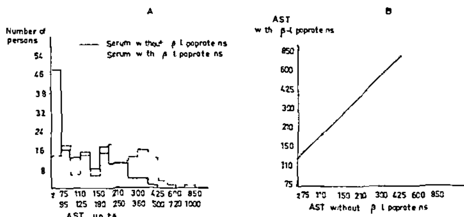


Fig 1

A The frequency of AST before and after depletion of β lipoproteins by dextran sulphate in 146 sera from blood donors
B Comparison of AST in the individual serum before and after depletion of β lipoproteins by dextran sulphate The plotted points represent the medians in the different classes The line corresponds to a decrease of 3/4 dilution step

donors as well as patients The serum samples from blood donors differed in some respects from those of in-patients (see below) The former were collected at any time during the day, often after a meal and were thus often lipaemic, whereas patient samples were taken in the morning before breakfast This is probably the reason why the primary AST values in blood donors were more widely dispered than in non icteric patients without any β haemolytic streptococcal disease

TABLE 1

The Median Values of AST in Different Serum Materials before and after Depletion of β Lipoproteins by Dextran Sulphate

Serum material	Number	AST with β lipoproteins	AST without β lipoproteins	Reduction in titre steps
Blood donors	146	146	210	1
Rheumatoid arthritis	194	125	75	¾
Haemolyt strepto coccal diseases Primarily ≧ 300 units	124	500	300	¾
Unclassified sera from routine investigations	675	180	95	1
Jaundice	180	720	110	2¾
Non icteric hypercholesterolemic sera	20	180	75	1¾

because of its striking influence on the AST of icteric sera and because of its simplicity. The dextran sulphate method was therefore chosen for the present investigation where we studied its capacity of reducing β lipoproteins in sera from healthy persons and patients with various diseases. Presumably, even healthy people have some amount of β lipoproteins capable of inhibiting SL O. The results of the electrophoretic studies mentioned above point in this direction. Rheumatoid arthritis is of special interest since it has been suspected that the relatively elevated AST that frequently occurs in this group represents a non specific factor.

METHODS AND MATERIALS

Antistreptolysin reaction.—Streptolysin from a broth culture of the S 84 strain was used. The broth was prepared from ox heart with 2 per cent peptone, 0.2 per cent glucose, 0.2 per cent $NaHCO_3$ and 0.1 per cent Na_2HPO_4, pH adjusted to 8.0. The streptolysin-containing broth was centrifuged and filtered through a Seitz filter. The amount of streptolysin units was determined by titration against standard anti streptoly…
The stand…
Institute
ml was c…
to a total volume of 1 ml per tube and was inactivated. 0.5 ml of streptolysin was added to each tube. After incubation of this mixture in a 37° C water bath for 15 minutes, 0.5 ml of a 5 per cent suspension of sheep erythrocytes was added. The mixture was then incubated at 37° C for 30 minutes and allowed to stand for 1 hour (or overnight) at 4° C before the degree of haemolysis was determined colorimetrically. The amount of antistreptolysin units present was calculated from the number of combined streptolysin units.

β lipoprotein precipitation.—The β lipoproteins were precipitated by the method of *Burstein & Samaille* (1958) in which 0.02 ml of 10 per cent dextran sulphate and 0.1 ml of 1M calcium chloride solution are added to 1 ml of serum. One hour later the mixture was centrifuged for 10 minutes at 1500 G. The clear supernatant was decanted and AST was determined.

Fresh sera were used. The AST values of untreated and of β lopiprotein depleted serum were determined at the same time.

Origin of the sera.—146 sera were obtained from blood donors taken to represent healthy adult individuals. 194 sera were from patients with rheumatoid arthritis who were all positive with regard to the rheumatoid factor. 124 sera with AST ≧ 300 units were from patients with acute tonsillitis, acute nephritis or rheumatic fever, i.e. patients supposed to be infected by β haemolytic streptococci and to have an elevated AST due to "real" antibody. Added to this were 675 sera from patients with non classified but non icteric diseases, 20 sera from patients with hypercholesterolaemia having cholesterol levels in serum of 400 mgs per cent or more, and finally 180 samples from 91 patients with jaundice from various causes recorded below.

RESULTS

The Decrease of AST in Different Sera Depletion of β Lipoproteins by Dextran Sulphate

Blood donors. The 146 blood donors had AST values ranging from very low titres up to 600 units (Fig 1). After depletion of the β lipoproteins the AST values fell about one dilution step and were decreased to 50 per cent of their previous numeric value. Table 1 shows the median AST values in sera with and without β lipoproteins from blood

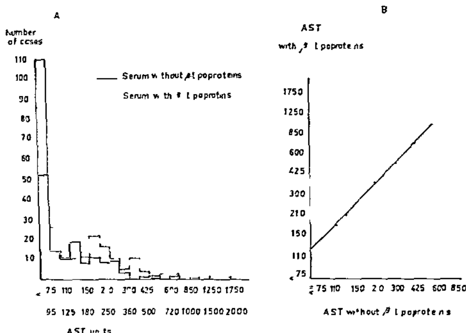


Fig 3

A The frequency of AST before and after depletion of β lipoproteins by dextran sulphate in 194 sera from patients with rheumatoid arthritis
B Comparison of AST in the individual serum before and after depletion of β lipo proteins by dextran sulphate The plotted points represent the medians in the different classes The line corresponds to a decrease of 3/4 dilution step

Unclassified diseases Excluded from this fairly heterogeneous group of 675 in patients were patients with rheumatoid, supposed β haemolytic streptococcal diseases with AST $\geq$ 300, and jaundic (Fig 4) The reduction of AST on depletion of β lipoproteins was similar to that found in the three previous groups The pre depletion median occurred in the 180 unit class and the post depletion median in the 95 unit class

Thus, the four groups of sera reported above would seem to possess fairly equal amounts of streptolysin inhibiting β-lipoproteins corresponding to 40–50 per cent of the primary AST value

Jaundice 180 serum samples from 91 cases of jaundice, bilirubin in serum 2 0 mg ml or more, were examined (Fig 5) Before depletion, these sera showed a wide range of AST values and very high values were frequently seen After depletion of the β lipoproteins, the AST values were markedly reduced and showed a distribution very similar to those of healthy people and patients without any haemolytic streptococcal disease The difference between the median values before and after treatment with dextran sulphate was much greater in this group than in the previous ones The pre-depletion median occurred in the 720 unit class and the post depletion median in the 110-unit class

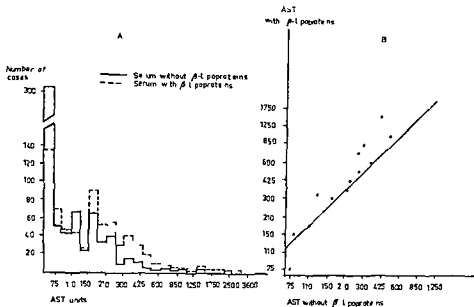


Fig 4

A The frequency of AST before and after depletion of β-lipoproteins by dextran sulphate in 675 sera from patients with non-classified non icteric diseases
B Comparison of AST in the individual serum before and after depletion of β-lipoproteins by dextran sulphate The plotted points represent the medians in the different classes The line corresponds to a decrease of 3/4 dilution step

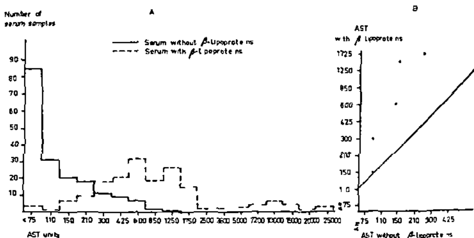


Fig 5

A The requency of AST before and after depletion of β-lipoproteins by dextran sulphate in 180 serum samples from 91 patients with jaundice from different causes
B *Comparison of AST in the individual serum* before and after depletion of β lipo proteins by dextran sulphate The plotted points represent the medians in the different classes The line corresponds to a decrease of 3/4 dilution step

The pre- and post depletion values of AST in one and the same icteric serum may show striking differences The medians for the different AST classes are plotted in Fig 5 B and will be seen to be far removed from the line representing a decrease of 3/4 dilution step Also illustrated by this figure is the slight tendency for a primarily very high AST not to drop to so low level as a primarily relatively low AST

TABLE 2

The Lack of Positive Relationship between the Amount of Bilirubin in Serum and the Extent to which AST Decreases on Depletion with Dextran Sulphate

Bilirubin/s	Decrease in AST, expressed in dilution steps following depletion of β lipoproteins						
	1	2	3	4	5	6	>6
> 16	3	4	6	6	3	3	5
14–16	3	4	1	5	3		
11–13	1	5	8	2	1		
8 10	3	9	16	8	5		
5– 7	6	19	20	10	4	1	3
2– 4		6	3	1			

The relationship between the decrease of AST on depletion of β-lipoproteins and the concentration of bilirubin is shown in Table 2 No correlation can be observed and, as will be further discussed below, there are sera that have a high bilirubin but show no abnormal elevation of AST Similarly, if crystallized bilirubin is diluted in normal serum the AST of this serum will not increase These results indicate that the streptolysin inhibiting β-lipoproteins are not identical with any substance connected to bilirubin itself but are probably derived from the injured liver in states of jaundice

The Relation of the Non specific, β Lipoprotein-Associated AST to different Clinical States of Jaundice

91 patients with jaundice were studied The main causes of jaundice in this material as listed in Table 3 were hepatitis, cirrhosis of the liver, biliary obstruction, and primary, or metastatic, malignant tumours of the liver It is of interest to note that sera from the only case studied of haemolytic jaundice and from patients who had recently undergone surgery of the biliary tract showed no abnormal elevation of AST in spite of pathological bilirubinaemia The same applies to one patient in the state of recovery from hepatitis The apparent absence of abnormal, non-specific AST in haemolytic jaundice is of great theoretical interest but further studies are needed to confirm it

In some cases where sera where examined at different stages of the disease, the non specific AST associated with abnormal levels of β-lipoproteins was found to appear later and disappear earlier than the

TABLE 3

Diagnosis in the Cases of Jaundice, Shown in Relation to Amount of Non Specific AST Depending on β-Lipoproteins

Diagnosis	Number of patients	AST before depletion of β-lipoproteins		Decrease in dilution steps	
		≧ 300	< 300	≧ $1\frac{1}{2}$	< $1\frac{1}{2}$
Hepatitis	14	13	1	12	2
Hepatitis in convalescent state	5	4	1	4	1
Lupoid hepatitis	1	1		1	
Toxical hepatosis	1	1		1	
Jaundice in pregnancy	1		1	1	
Haemolytic jaundice	1		1		1
Jaundice of unknown origin	1	1		1	
Cirrhosis of the liver	16	16	1	16	1*
Cancer of the pancreas Choledocholithiasis Cholecystitis	29	27	2	28	1
Cancer of the liver Cancer of the gallbladder	8	8		7	1
Metastatic cancer in the liver	11	9	2	8	3*
Diseases in the gallbladder, operated and in convalescent state	3		3		3

* Different results in one patient

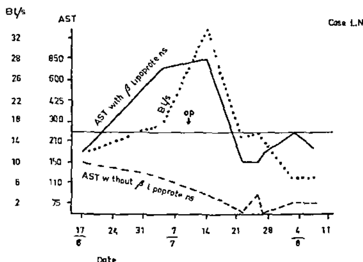


Fig 6

The variations of bilirubin and specific and non specific AST in a case of cholecysto and choledocholithiasis with jaundice, cured by lithotomy

pathological amount of bilirubin A few definitions are due in this connexion The AST was considered to be elevated if it was more than 300 units, and "abnormal" β-lipoproteins to be manifested by a depression of the AST by more than 2 dilution steps Fig 6 illustrates a case of cholecysto- and choledocholithiasis where, in spite of bilirubinemia, the AST at the early and late stages was not markedly influenced by abnormal β-lipoproteins

TABLE 4

Diagnoses in the Studied Cases of Hypercholesterolaemia without Jaundice Shown in Relation to Non-Specific AST, Due to β Lipoproteins

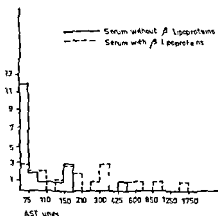
Diagnosis	Number of patients	Patients with abnormal AST due to β lipoproteins	Patients without abnormal elevated AST
Cardio arteriosclerosis	10	3	7
Xanthomatosis	3	1	2
Hypothyreosis	3		3
Diabetes mellitus	2		2
Lipoid nephrosis	2	2	

Hypercholesterolemia 21 cases of non icteric hypercholesterolaemia with different diagnoses are listed in Table 4 and the pre- and post-depletion AST values of these sera are shown in Fig 7 Some of these sera had a high primary AST which was markedly reduced on repletion with dextran sulphate, but the majority 'ordinary" AST values The reduction as expressed in dilution steps was about 1¼ The pre-depletion median occurred in the 180 unit class, the post depletion median in the 75 unit class

Abnormal AST values associated with β lipoproteins were observed in a few of these sera, namely one case of lipoid nephrosis, in 3 of the 10 cases of cardio-arteriosclerosis, and in one case of xanthomatosis (Table 4) The other cases did not show any abnormal AST However, since this material consisted of only 21 patients it does not permit any definite conclusions concerning the occurrence of streptolysin-inhibiting β lipoproteins in hypercholesterolaemia

Sera with a Monoclonal, Abnormally Elevated AST

Among sera, obtained from patients with myeloma and essential, benign hyperglobulinaemia, described elsewhere (*Waldenstrom, Winblad, Hallen & Liungman*), we found three cases of monoclonal AST reaching values of 1/512000, 1/8000, and 1/16000, respectively These titres were not decreased on depletion with dextran sulphate and thus did not depend on β-lipoproteins



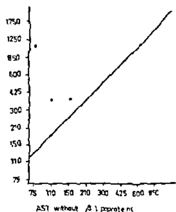


Fig 7

A The frequency of AST before and after depletion of β lipoproteins by dextran sulphate in 21 sera from patients with non icteric hypercholesterolemia

B Comparison of AST in the individual serum before and after depletion of β lipoproteins by dextran sulphate The plotted points represent the medians in the different classes The line corresponds to a decrease of 3/4 dilution step

DISCUSSION

The dextran sulphate method for depleting serum of β lipoproteins seems well suited to studies of the non-specific AST connected with β-lipoproteins Such non-specific AST is especially prominent in icteric and hypercholesterolaemic sera Other sera, including those of healthy individuals, show a fairly constant level of "ordinary" β lipoprotein Our study indicates that about 50 per cent of the AST value is due to such β lipoprotein inhibiting the streptolysin The remaining 50 per cent is to be considered as the real antibody

It may be argued that the protein of this β-lipoprotein be an antibody in spite of its lipid connexion We do not think this in the case The high levels of β-lipoproteins and "non-specific" AST in sera from patients with jaundice were not associated with any known infections of β-haemolytic streptococci In addition, the frequency of true, non lipid AST in such icteric sera was the same as in sera from healthy people

When an AST of 300 units or more is found in a serum, the clinician will be interested to know if it represents real antibody but also wether it is due to a higher than normal concentration of β lipoproteins A verification test using dextran sulphate may then be of value making it possible to compare pre- and post depletion AST values In routine work, one possibility is to subject all sera to such depletion

but the alternative practiced in our laboratory is to perform verification tests only on sera with a primary AST of 300 units or more

Another point of interest to the clinician may be to follow the changing levels of β lipoproteins in cases of jaundice This can be done by direct estimation of the β lipoproteins but may also be expressed in terms of non specific AST Our investigations have demonstrated abnormal amounts of AST in nearly all cases of jaundice Exceptions were the only case studied of haemolytic jaundice as well as early and late stages of hepatitis and jaundice due to biliary obstruction where the discrepant findings of high bilirubin and low non specific AST may be of interest in convalescent stages of the disease

SUMMARY

The dextran sulphate method for depleting serum of β lipoproteins associated with non specific AST has proved to be very useful and well suited for routine work The normal level of such β lipoproteins as studied in sera from 146 blood donors has been found to be about 40 50 per cent of the numeric value of AST units

In sera from patients with β haemolytic streptococcal diseases with AST $\geq$ 300 units the depleting effect of dextran sulphate was the same with rheumatoid arthritis (sero positive) and sera from non icteric patients with unclassified diseases

Very high values of AST were seen in nearly all the sera from patients with jaundice Depletion with dextran sulphate caused a reduction of AST to values similar to those seen in states of health and in non streptococcal diseases The high level of non specific AST associated with β lipoproteins was common to nearly all forms of jaundice whether due to hepatitis biliary obstruction or malignant disease of the liver It was not seen however in the only studied case of haemolytic jaundice nor was it found in early or convalescent stages of liver disease

A few cases of non icteric hypercholesterolemia showed pathological amounts of non specific AST but the majority were normal

Three cases of monoclonal abnormally elevated AST in myelomatosis and macroglubulinaemia were examined The amount of non *specific AST in these sera was normal*

In routine work it is recommended to assay the amount of non specific AST associated with β lipoproteins in sera with "elevated AST using the dextran sulphate method

REFERENCES

Bernfeld P Nisselbaum J S Berkeley B J & Hanson R W The Influence of chemical and physicochemical nature of macromolecular polyanions on their interaction with human serum β lipoproteins J Biol Chem 235 2857 1960

Biol (Paris) *157* 949 1963
Hedberg H The specific antistreptolysin activity in subfractions of human gamma globulins Comunicazione al X Congresso della Lega Internazionale contro il Reumatismo 1961
Hedberg H & Moritz U The antistreptolysin activity in chromatographic fractions of serum and subfractions of γ globulin preparations Acta Rheum Scand *6* 179 1960
Hewitt L F & Todd E W The effect of cholesterol and of sera contaminated with bacteria on the haemolysins produced by haemolytic streptococci J Path Bact *49* 45 1939
Hallén J Non specific streptolysin O inhibition in diseases of the liver and biliary system Acta path et microbiol scandinav *57* 301 1963
Killander J & Philipson L Separation of rheumatoid factors and antistreptolysins by Gel filtration and preparative electrophoresis Acta path et microbiol scandinav *61* 127 1964
Legler F Berg G Scheiffarth F & Leder R Untersuchungen uber den Einfluss leichter Lipoproteide auf den Ausfall der Antistreptolysin Reaktion Z Rheumaforsch *20* 440 1961
Oker Blom N Mikkila E & Kalaja T Non specific antistreptolysin reactions in hepatitis sera Antistreptolysin titers before and after removal of lipids from the hepatitis sera with bentonite Ann Med exp Fenn *28* 125 1950
Packalen T Nonspecific antistreptolysin reactions and serum (or pleural exudate) cholesterol J Bact *56* 143 1948
Scheiffarth F Berg G Legler F & Petersmann G Untersuchungen uber die Bedeutung der Serumlipoide fur den Antistreptolysintiter Klin Wschr *35* 412 1957
Waldenström J Winblad S Hallén J & Liungman S The occurrence of serological Antibody reagins or similar γ globulins in conditions with monoclonal hypergammaglobulinemia such as myeloma macroglobulinemia etc Acta Med Scand *176* 619 1964

Acta path et microbiol scandinav 66 105–112 1966

The Bacteriological Laboratory Ullevål Hospital Oslo Norway

BACTERIOPHAGE TYPING OF *STAPHYLOCOCCUS AUREUS* USING A SINGLE CONCENTRATION OF PHAGE (100 × RTD)

By

MIKLOS DEGRÉ

Received 10 vii 65

Typing of *Staphylococcus aureus* cultures by bacteriophage is a recognized method The method was described by *Fisk* (1942) who was the first to use a solid medium, thereby putting phage specificity into practical use In his work he used undiluted solutions of phage This led to numerous difficulties and errors Concentrated solutions may contain not only the propagated phage, but also other temperated phages from the propagating strain (*Rountree*, 1949) This error can be eliminated by dilution *Williams & Rippon* (1952) described that undiluted solutions of phage might give inhibition reactions, some of which would be difficult to separate from true confluent lysis This phenomenon has later been described several times by other workers The use of concentrated phage solution may result in a too high phage-bacteria ratio, which may result in lysis from-without (*Ralston et al* 1957)

Wilson & Atkinson (1945) were the first to suggest the use of Routine Test Dilution (RTD), *i e* the highest dilution giving confluent lysis on its propagating strain This method was adapted from bacteriophage typing of S typhi (*Cragie & Yen* 1938) The use of RTD meant a great improvement and the method has been little altered since

Ever since the early days of bacteriophage typing it has been known that certain Staph aureus strains did not show lysis using the recommended phage strains at their RTD The frequency of these nontypable (NT) strains seems to have increased although several new phages have been included in the general routine Neither does the use of phage-pools with several less common phage strains seem to eliminate a great number of NT strains *Williams & Rippon* (1952) were the first to try to retype these strains with more concentrated phage solutions They have used undiluted solutions at first Later this was substituted by 1000 times RTD to avoid false reactions (*Rippon* 1956)

This study has been supported by a grant from Oslo Kommune

By this method it was possible to type 85–95 per cent of the isolated Staph aureus strains

The use of 1000 × RTD, however, gave rise to further problems, mainly inhibition reactions, sometimes quite strong ones These may often be specific for the individual strains, but are difficult to ascertain Being a relatively concentrated phagedilution, 1000 × RTD often gives more complicated and somewhat less specific patterns than RTD It is an important disadvantage to have to operate with two different concentrations The pattern of reaction of typing is performed with 1000 × RTD can hardly be compared with the pattern obtained if just RTD is used A large percentage of strains are only typable at 1000 × RTD (often 30 per cent or more) One is then dealing with two separate main groups, the patterns of which are based on different principles Most strains belong definitely to one of the groups typable at RTD or typable at 1000 × RTD Some strains, however, take up an intermediate position, sometimes they are typable at RTD, at other times only at 1000 × RTD Small variations in the media used for typing or in the strains themselves, may be sufficient to bring about such a change

It would be desirable to find a concentration allowing phage typing of approximately as many strains as when conventional methods are used, and simultaneously reducing the number of false reactions Avoiding typing with two concentrations would obviously alleviate the amount of work involved *Wahl & Lapeyre-Mensignac* (1950) suggested that a phage solution more concentrated than RTD would be preferable *Wahl & Fouace* (1954) thought that a concentration between 10 and 100 × RTD would be the best substitute for the usual concentrations *Zierdt & March* (1962) arrived at the same conclusion Inhibition reactions have hardly been noticed at this strength In relation to RTD a further 14 6 per cent strains (total 81 3 per cent) were amenable to typing In this particular experiment 1000 × RTD was not used The reproducibility of types seemed to increase when 100 × RTD was used *Pohn* (1957) has found 26 per cent more typable strains at 100 × RTD than at RTD, but 13 per cent less than at 1000 × RTD The typing patterns were not less specific at the higher concentrations

This work is an attempt to show that using a single concentration (100 × RTD) of phage one can type practically as many of the isolated Staph aureus strains as by conventional methods, without losing type specificity and without false reactions

MATERIAL AND METHODS

In the routine typing 24 phages were used Of these 21 belong to the basic set recommended by the Subcommittee of Phage Typing of *Staphylococcus* (*Blair & Williams* 1961) Besides 83A 83B (*Blair & Carr*) and KS6 (*Wallmark*) were included For practical purposes 81 and KS6 were registered in group I

In propagation and testing the principles outlined by *Blair & Williams* 1961)

were followed The soft agar method was usually preferred for propagation occa sionally the broth method was used The page solutions were filtered through Seitz filter and stored at +4° C For typing 4 hour-cultures were used The cultures were poured into agar dishes which dried for 30 minutes at room temperature before the phages were added The phages were applied by means of a semiautomatic multiple

No phage was used undiluted The phages were controlled weekly on their pro pagating strain

All reactions were registered by the number of plaques (Pl) up to 50 Pl

Grading followed the usual principles ++ confluent lysis and 50 or more Pl + 20–50 Pl ± 1 20 distinct Pl Registered inhibition reactions were graded according to *Zierdt & March* (1962) complete diffuse inhibition incomplete diffuse inhibition and concomitant inhibition and true lytic reactions Complete diffuse inhibition gave obscure readings as they were very diff cult to distinguish from true confluent lysis In such cases the type of reaction could be determined only by a comparison of different concentrations

The material consisted of 400 coagulase positive strains received for routine examination from departments and outpatient cl nics within Ullevål Hospital The strains were typed in succession and without selection using RTD and 100 × RTD Strains not showing ++ reactions at RTD were retyped the following day at 1000 × RTD There was no epidem c situation in the hospital during the collection of the samples

TABLE 1

Typing Reactions of 400 Staph aur *Cultures at RTD 100 × RTD and 1000 × RTD*

	RTD	1000 × RTD	Total	100 × RTD
Typable cultures (Major reactions)	191	149	340	302
Percent of total cultures	47 75	37 25	85 0	75 5
Minor reactions only	68	23		46
Nontypable cultures	141	37		52
Percent of total cultures	35 25	9.25		13 0

RESULTS

Table 1 shows the total number of typing reactions on 400 *Staph aureus* strains using RTD and 1000 × RTD as well as 100 × RTD 340 strains were typable (85 per cent) by the conventional methods counting ++ reactions only In addition there were 7 (1 75 per cent) + reactions at 1000 × RTD which is also routinely reported 191 of these strains showed reactions at RTD In 209 cases (52 25 per cent) it was necessary to retype with 1000 × RTD Using the conventional phage set 37 strains (9 25 per cent) showed no true lytic reactions at all

At 100 × RTD 302 strains gave ++ lytic reactions Using the con ventional concentrations thus yielding positive reactions in 9 2 per cent

By this method it was possible to type 85 95 per cent of the isolated Staph aureus strains

The use of 1000 × RTD, however, gave rise to further problems mainly inhibition reactions sometimes quite strong ones These may often be specific for the individual strains but are difficult to ascertain Being a relatively concentrated phagedilution, 1000 × RTD often gives more complicated and somewhat less specific patterns than RTD It is an important disadvantage to have to operate with two different concentrations The pattern of reaction of typing is performed with 1000 × RTD can hardly be compared with the pattern obtained if just RTD is used A large percentage of strains are only typable at 1000 × RTD (often 30 per cent or more) One is then dealing with two separate main groups the patterns of which are based on different principles Most strains belong definitely to one of the groups typable at RTD or typable at 1000 × RTD Some strains, however take up an intermediate position, sometimes they are typable at RTD, at other times only at 1000 × RTD Small variations in the media used for typing or in the strains themselves may be sufficient to bring about such a change

It would be desirable to find a concentration allowing phage typing of approximately as many strains as when conventional methods are used, and simultaneously reducing the number of false reactions Avoiding typing with two concentrations would obviously alleviate the amount of work involved *Wahl & Lapeyre Mensignac* (1950) suggested that a phage solution more concentrated than RTD would be preferable *Wahl & Fouace* (1954) thought that a concentration between 10 and 100 × RTD would be the best substitute for the usual concentrations *Zierdt & March* (1962) arrived at the same conclusion Inhibition reactions have hardly been noticed at this strength In relation to RTD a further 14 6 per cent strains (total 81 3 per cent) were amenable to typing In this particular experiment 1000 × RTD was not used The reproducibility of types seemed to increase when 100 × RTD was used *Pohn* (1957) has found 26 per cent more typable strains at 100 × RTD than at RTD but 13 per cent less than at 1000 × RTD The typing patterns were not less specific at the higher concentrations

This work is an attempt to show that using a single concentration (*100 × RTD*) of phage one can type *practically as many of the isolated Staph aureus strains* as by conventional methods, without losing type specificity and without false reactions

MATERIAL AND METHODS

In the routine typing 24 phages were used Of these 21 belong to the basic set recommended by the Subcommittee of Phage Typing of *Staphylococcus* (*Blair & Williams 1961*) Besides *83A 83B* (*Blair & Carr*) and KS6 (*Wallmark*) were included For practical purposes 81 and KS6 were registered in group I

In propagation and testing the principles outlined by *Blair & Williams* (1961)

were followed The soft agar method was usually preferred for propagation, occasionally the broth method was used The page solutions were filtered through Seitz filter and stored at +4° C For typing, 4 hour-cultures were used The cultures were poured into agar dishes which dried for 50 minutes at room temperature before the phages were added The phages were applied by means of a semiautomatic multiple-

[illegible] ilution pro[illegible] strain The titre in use was generally [illegible] of 5×10^3 No phage was used undiluted The phages were controlled weekly on their propagating strain

[illegible]

true confluent lysis In such cases the type of reaction could be determined only by a comparison of different concentrations

The material consisted of 400 coagulase positive strains received for routine examination from departments and outpatient clinics within Ullevål Hospital The strains were typed in succession and without selection using RTD and 100 × RTD Strains not showing ++ reactions at RTD were retyped the following day at 1000 × RTD There was no epidemic situation in the hospital during the collection of the samples

The strains were registered in groups following the usual methods Type 80/81 was registered separately In this type were included 52/52A/80/81, 80/81/KS6 and 52 52A 80/81/KS6 Only strains showing no true lytic reaction were registered as non typable (NT)

TABLE 1

Typing Reactions of 400 Staph aur *Cultures at RTD 100 × RTD and 1000 × RTD*

	RTD	1000 × RTD	Total	100 × RTD
Typable cultures (Major reactions)	191	149	340	302
Percent of total cultures	47 75	37 25	85 0	75 5
Minor reactions only	68	23		46
Nontypable cultures	141	37		52
Percent of total cultures	35 25	9 25		13 0

RESULTS

Table 1 shows the total number of typing reactions on 400 *Staph aureus* strains using RTD and 1000 × RTD, as well as 100 × RTD 340 strains were typable (85 per cent) by the conventional methods counting + + reactions only In addition there were 7 (1 75 per cent) + reactions at 1000 × RTD which is also routinely reported 191 of these strains showed reactions at RTD In 209 cases (52 25 per cent) it was necessary to retype with 1000 × RTD Using the conventional phage set, 37 strains (9 25 per cent) showed no true lytic reactions at all

At 100 × RTD 302 strains gave + + lytic reactions Using the conventional concentrations thus yielding positive reactions in 9 2 per cent

more cases than 100 × RTD By including 25 + reactions the figure is higher by 20 (5 per cent) if the conventional double typing is used than if 100 × RTD alone is used Table 2 summarizes the minor reactions at RTD 1000 × RTD and 100 × RTD

TABLE 2

Minor Lytic Reactions of 400 Staph aureus *Strains at RTD 100 × RTD and 1000 × RTD*

Lytic reactions	RTD	1000 × RTD	100 × RTD
20 50 Pl	19	17	25
1 50 Pl	49	16	21

TABLE 3

Distribution of 400 Staph aureus *Cultures on the Different Phage Groups at RTD 1000 Times RTD and at 100 Times RTD*

	Concentration used for typing	Type 80/81	Group I	Group II	Group III	Group IV & V	Misc	Total
Typable at RTD	RTD	93	22	27	39	4	6	191
	100 × RTD	94	12	23	9	4	49	191
Typable at 1000 × RTD	1000 × RTD	12	14	4	42	0	77	149
	100 × RTD	10	16	5	47	0	33	111
Total	RTD and 1000 × RTD	103	40	31	81	4	83	340
	100 × RTD	104	28	28	57	4	82	302

Only major reactions included

Table 3 shows the distribution of typable strains on the different phage groups with the three concentrations The total distribution of patterns is roughly the same at 100 × RTD as at RTD and 1000 × RTD The only significant displacement is seen between group III and Miscellaneous At higher concentrations complicated types and mixed groups are more frequently encountered The total number of strains in the miscell group however is practically the same at 100 × RTD as at the conventional concentrations (82 versus 83)

The table does not indicate how often a change is seen when the pattern obtained when the conventional concentrations are used compared with the pattern obtained at 100 × RTD nor the degree of such change if present Of the 191 strains typable at RTD complete agreement in pattern was seen in 67 cases In 79 strains the pattern at 100 × RTD was somewhat more complicated some extra + + reactions being involved here In most of these strains there were alterations of type 3A + to 3A 3B 3C and types 80 81 or 81/KS6 to 52 52A 80 81 KS6 In all these cases the strains have remained in their original groups In 45 strains however the reactions were altered with several new lytic

reactions which influenced upon the distribution on groups Most of these originated in group III, and had additional reactions with phage 79, KS6 or 80 81 which classify them in the miscellaneous group

The corresponding figures for strains typable at 1000 × RTD were with identical results, 24 strains, somewhat more complicated patterns within the original group without change in group, 54 strains, and in 33 cases alteration of group at 1000 × RTD as compared with 100 × RTD

Table 4 shows the number of strains with false lytic reactions at different phage concentrations For each strain the strongest inhibition reactions only have been included in this table In one case complete inhibition was registered with all three concentrations Typing at 0 1 × RTD yielded no reaction in this case

TABLE 4

False Lytic Reactions at RTD 100 Times RTD and 1000 Times RTD on 400 Staph aureus *Cultures*

False lytic Reactions	RTD	100 × RTD	1000 × RTD
Complete diffuse inhibition	1	1	32
Incomplete diffuse inhibition	0	12	22
Concomitant true and false reactions	1	13	25

DISCUSSION

Any method of typing attempts to fulfil two requirements, a) giving reproducible specific types, and b) as many strains as possible should be typable Often the one may derive benefit at the expense of the other This is for instance so in bacteriophage typing of Staph aureus By using two dilutions, RTD and 1000 × RTD, a compromise had been reached, whereby the two different concentrations each satisfy one part of the requirements RTD gives specific reactions, *but* only 40–70 per cent of the strains typable, whereas typing at 1000 × RTD often gives non specific reactions (*Blair & Carr,* 1960) but allows typing of 85 95 per cent of the strains The routine use of such different concentrations however, provides in many cases results which are not reproducible and not comparable The introduction of a single concentration might satisfy the requirements better

The use of 100 × RTD seems to be a promising compromise As expected a greater number of strains (17 75 per cent) were tyable at 100 × RTD than just at RTD Retyping at 1000 × RTD gives a further 9 5 per cent tyable strains

The number of strains typable at 100 × RTD could probably be extended further Moderate reactions (+) were frequently seen without ++ reactions Moderate reactions at RTD were not considered

to be reportable It is partly because they are not as well reproducible as stronger reactions and partly because practically *all* of these reappear at 1000 × RTD as strong reactions This is also the case of moderate reactions at 100 × RTD All of these (25) reappeared at 1000 × RTD as + + reactions These + reactions at 100 × RTD seem to be more specific and reproducible than at 1000 × RTD and ought to be included in routine reports where they appear alone if typing is carried out with 100 × RTD only This is not the case of weak (±) reactions which do not seem to be sufficiently stable They may easily disappear when they are small variations in the media etc A fair number of these + strains (22 25) gave + reactions with 30 50 Pl This might indicate that 30 50 Pl ought to be reported as weak reactions only However the material is much too scanty for definite conclusions to be drawn

By this method using + reactions appearing alone as well as + + reactions at 100 RTD 81 82 per cent of the 400 strains were typable which is not a significantly lower percentage than the percentage of typable strains obtained at RTD and 1000 × RTD

Leaving out typing at 1000 × RTD the majority of inhibition reactions will be excluded particularly those giving complete diffuse inhibition This compares well with the results obtained by *Zierdt & March* (1962) The majority of inhibition reactions were seen in group III usually several within the same strain As mentioned none of these phage solutions had to be diluted less than 10 times to reach 1000 × RTD No diffuse inhibition was seen with the phage 3B which was only diluted 5 times to reach 1000 × RTD The degree of dilution is probably not the only deciding factor in producing these false reactions

It is a definite advantage to work with well defined types such as S typhi phage types The typing of Staph aureus on the other hand is based on different lytic reactions forming a variety of patterns Patterns made up by numerous reactions might well be an indication of less specificity This is probably even more pronouced with lytic reactions within different groups Williams (*Anderson & Williams* 1956) assumed that higher concentrations e g 100 × RTD would complicate the types far too much to be of use in routine work Comparing the results obtained by 100 × RTD and by the conventional methods however this does not seem to be a major problem It does give somewhat more complicated patterns than at RTD but simpler than at 1000 × RTD (which just about cancels any displacement) Altogether we found 25 strains with minor and 12 strains with major alterations at 100 × RTD compared to findings using the conventional concentrations One would avoid some extremely complicated patterns which are sometimes seen at 1000 × RTD *Pohn* (1957) has found the same phages in group III involved in these atypical reactions He suggests not to base group classification on these phages

Zierdt & March (1962) have found a marked reduction of weak (+ and ±) reactions at 100 × RTD as compared to RTD This reduction we have not been able to confirm The number of weak reactions seem to increase in proportion to increased phage concentrations and to the number of strong reactions There is a very constant ratio of typable strains to strains with weaker reactions 1 05 1 10 and 1 12 at RTD, 100 × RTD and 1000 × RTD, respectively The number of weaker reactions for each typable strain is also quite constant at different concentrations (2 8 2 6, and 2 2)

By substituting the conventional phage concentrations (RTD and 1000 × RTD) with a single concentration (100 × RTD) the loss seems to be slight compared to the gain There would be 3–4 per cent fewer typable strains and approximately 10 per cent of the typable strains would give a slightly more complicated pattern as compared to the ones obtained by the conventional methods On the other hand one could exclude retyping with stronger concentration, to avoid to operate with 2 groups of "types' which are not easily comparable One would also avoid the occurrence of all major false reactions as well as the majority of less significant ones

SUMMARY

400 cultures of *Staphylococcus aureus* were typed with the recommended phage set by conventional method, at RTD and at 1000 × RTD The results were compared to typing results obtained at 100 × RTD By using the 100 × RTD concentrations 81–82 per cent of strains could be typed as compared with 47 75 per cent obtained at RTD, and 86 75 per cent at 1000 × RTD Some few cultures had a more complicated pattern if typed at 100 × RTD as compared with the conventional concentrations The false lytic reactions which are numerous at 1000 × RTD are reduced at 100 × RTD Only slight degrees of inhibition reactions were seen at 100 × RTD The reproducibility of the 'types' seems to be increased at 100 × RTD, but this object needs more examination

REFERENCES

1 *Anderson E S & Williams R E O* Bacteriophage typing of enteric pathogens and staphylococci and its use in epidemiology J Clin Pathol *9* 94 127 1956

2 *Blair J E & Carr M* The techniques and interpretation of phage typing of staphylococci J Lab Clin Med 55 650–662 1960

3 *Blair J F & Williams R E O* Phage typing of staphylococci Bull World

4 *Craig*

5 *Fisk*

among strains of *Staphylococcus aureus* J Infect. Dis *71* 153–160 1942

6 *Fisk R T* Studies on Staphylococci Identification of *Staphylococcus aureus* strains by means of bacteriophage J Infect Dis *71* 161 165 1942

to be reportable It is partly because they are not as well reproducible as stronger reactions and partly because practically *all* of these reappear at 1000 × RTD as strong reactions This is also the case of moderate reactions at 100 × RTD All of these (25) reappeared at 1000 × RTD as ++ reactions These + reactions at 100 × RTD seem to be more specific and reproducible than at 1000 × RTD, and ought to be included in routine reports where they appear alone if typing is carried out with 100 × RTD only This is not the case of weak (±) reactions which do not seem to be sufficiently stable They may easily disappear when they are small variations in the media etc A fair number of these + strains (22/25) gave + reactions with 30–50 Pl This might indicate that 30–50 Pl ought to be reported as weak reactions only However, the material is much too scanty for definite conclusions to be drawn

By this method using + reactions appearing alone as well as ++ reactions at 100 RTD, 81–82 per cent of the 400 strains were typable, which is not a significantly lower percentage than the percentage of typable strains obtained at RTD and 1000 × RTD

Leaving out typing at 1000 × RTD the majority of inhibition reactions will be excluded, particularly those giving complete diffuse inhibition This compares well with the results obtained by *Zierdt & March* (1962) The majority of inhibition reactions were seen in group III, usually several within the same strain As mentioned none of these phage solutions had to be diluted less than 10 times to reach 1000 × RTD No diffuse inhibition was seen with the phage 3B which was only diluted 5 times to reach 1000 × RTD The degree of dilution is probably not the only deciding factor in producing these false reactions

It is a definite advantage to work with well defined "types" such as S typhi phage-types The typing of Staph aureus on the other hand, is based on different lytic reactions forming a variety of patterns Patterns made up by numerous reactions might well be an indication of less specificity This is probably even more pronouced with lytic reactions within different groups Williams (*Anderson & Williams 1956*) assumed that higher concentrations *e g* 100 × RTD would complicate the types far too much to be of use in routine work Comparing the results obtained by 100 × RTD and by the conventional methods, however, this does not seem to be a major problem It does give somewhat more complicated patterns than at RTD, but simpler than at 1000 × RTD (which just about cancels any displacement) Altogether we found 25 strains with minor and 12 strains with major alterations at 100 × RTD compared to findings using the conventional concentrations One would avoid some extremely complicated patterns which are sometimes seen at 1000 × RTD *Pohn* (1957) has found the same phages in group III involved in these atypical reactions He suggests not to base group-classification on these phages

Acta path et microbiol scandinav 66, 113–123 1966

The State Veterinary Medical Institute, Helsinki Finland and the State Veterinary Medical Institute, Stockholm Sweden

ISOLATION OF SEP AGENT FROM PIGS WITH ENZOOTIC PNEUMONIA IN FINLAND

By

T Estola and A Schulman

Received 9 vi 65

Swine enzootic pneumonia occurs at a fairly high frequency in Finland and has to be attributed considerable significance as a factor causing lowered production both in piggeries producing piglets and in those where slaughter pigs are raised

Prevention of the disease is waged by the Finnish State, in order to combat the disease more effectively a continuous health observation programme covering all pig breeding establishments has been in operation since the beginning of 1964

These countermeasures, which require reliable diagnostic methods, have so far been based on clinical examination as well as pathological examination of the respiratory organs At clinical examination the main attention has been centered on cough However, the result of such an examination may be indicative at most because obviously all cases of cough cannot be due to enzootic pneumonia, on the other hand cases are encountered in which infected individuals display no clinical symptoms

Neither can a definite diagnosis be arrived at by macro-pathological and histological findings since, as has been stated in several studies (*Hjärre et al* 1952, *Hjarre* 1957, *Goodwin & Whittlestone* 1960, *Obel* 1961 *Bjorklund* 1963), no pathological changes entirely specific of the disease are manifest in association with enzootic pneumonia Changes identical to those observed in enzootic pneumonia can be produced, for instance, with type A influenza virus, which causes influenza in pigs in USA (*Hjärre et al* 1952)

In Sweden, tissue cultures, embryonated hens eggs and PPLO sub strates have been successfully employed to isolate an agent in cases

---

This work was partly aided by a grant from *the Medical Science Commission of the Finnish State*

The authors wish to thank Dr *K Bakos* of State Veterinary Medical Institute Stockholm Sweden for the SEP agent he kindly placed at their disposal and for the *Mycoplasma granularum* and *Staphylococcus aureus* strains For histopathological examination of the material we are obliged to Mrs *Anna-Stina Garry Andersson* Veterinarian of the State Veterinary Medical Institute Helsinki

of enzootic pneumonia, which is called the SEP (swine enzootic pneumonia) agent The present experiments were undertaken with a view of isolating this agent also in Finland The primary object was to adopt, if possible, a procedure of diagnosis in respect of swine enzootic pneumonia which was based on isolation of the disease-producing organism

The literature dealing with enzootic pneumonia is rather extensive Summarizing the previous literature, it can be said that since the investigations of *Kobe*, published in 1933, in which he distinguished enzootic pneumonia from the swine influenza described by *Shope* (1931), up to 1954 (*Terpstra* 1935, *Momberg-Jorgensen* 1938, *Slagsvold* 1942, *Gulrajani & Beveridge* 1951, *Betts* 1952, *Hjarre et al* 1952, *Beveridge & Betts* 1953, *Dinter et al* 1954) enzootic pneumonia was treated as a virus disease or as a disease caused by a virus and bacteria (in the first place *Haemophilus influenzae suis*) in combination

During this period the first investigations of enzootic pneumonia were also carried out in Finland (*Rislakki* 1953, *Penttinen & Rislakki* 1953), in which the transfer of enzootic pneumonia from one piglet to another with bacteriafree material was successfully accomplished but the disease producing agent could not be made to multiply in embryonated eggs

In 1954, isolation of a cytopathogenic agent by the tissue culture from lung suspension of pigs affected with enzootic pneumonia was successfully carried out in Sweden (*Wesslen & Lannek* 1954), the agent proving sensitive to broad-spectrum antibiotics In stained preparations this agent could also be recognized with a light microscope (*Lannek & Wesslen* 1955) It was called the SEP agent When piglets were experimentally inoculated with it, only microscopic changes were found (*Lannek & Wesslén* 1957)

Soon afterwards, the cultivation of the SEP agent in the yolk sac of embryonated hens' eggs was also achieved (*Dinter et al* 1957) *Lannek & Wesslen* (1957) had already suspected that the SEP agent might be a mycoplasma (PPLO) species, but this was not confirmed until it could be made to grow on PPLO substrate (*Bakos et al* 1962, *Bakos & Dinter* 1963) In continued studies by *Dinter et al* (1965) the SEP agent proved to be closely related with a mycoplasma species previously described in USA (*Switzer* 1953), and there given the name *Mycoplasma hyorhinis* (*Switzer* 1955), but it had not been associated with the aetiology of enzootic pneumonia (*Roberts et al* 1962) or had been considered a secondary infection in this disease (*L'Ecuyer & Switzer* 1963)

In the most recent publications by Bakos and Dinter (*Bakos & Dinter* 1963, *Dinter* 1964) they drew the conclusion that studies up to date already justify the assumption that the SEP agent is identical with the aetiological factor of enzootic pneumonia Their conjecture is supported by other recently published reports on the isolation of SEP

agent in cases of enzootic pneumonia e g in Japan (*Takatori et al* 1964) and in Germany (*Monreal et al* 1964)

MATERIAL

The present material comprises 31 swine lungs and 26 nasal mucosa samples derived from 31 animals and from 27 piggeries all of them pig producing establishments and distributed all over Finland The material was in part gathered personally by the authors in part it was taken from autopsy material sent to the State Veterinary Medical Institute

In the first mentioned instances the pigs selected on the strength of clinical examination were transported to the State Veterinary Medical Institute either alive or immediately after killing and the actual sampling was carried out at the Institute within 24 hours If the piggery was situated at a great distance from the Institute the samples were taken after autopsy performed on the spot and brought to the laboratory in thermos flasks in refrigerated condition

From autopsy materials only the fresh cases were chosen in which the animal had been dead no longer than 1 2 days

The samples were taken from 22 animals with suspected enzootic pneumonia on the basis of clinical examination and/or macroscopic changes observed at autopsy and from 9 animals in which no infection by enzootic pneumonia was suspected on the strength of the above mentioned examinations The parts of the lungs displaying changes were used for the isolation tests From lungs of normal macroscopic appearance samples were taken from the apical and cardiac lobes With part of the material analogous isolation tests were carried out on the nasal mucosa All samples were moreover subjected to standard histopathological examination

METHODS

The isolation tests were mainly carried out by the methods established by Dinter and Bakos (*Dinter et al* 1957 *Bakos et al* 1962 *Bakos & Dinter* 1963) involving inoculation of embryonated hens eggs and of PPLO substrates

Inoculation of embryonated hens eggs The samples were cut into pieces with scissors and subsequently ground in a mortar Of the homogenized tissue mass a 20 per cent suspension was prepared with phosphate buffer (pH 7 4) or with physio logical saline

The suspension was then centrifuged for 15–20 minutes at 2000 r p m The super natant was treated with penicillin (10 000 I U per ml) and streptomycin (10 mg per ml)

If the sterility test on blood agar failed to produce growth the suspension was injected through the air cell into the yolk sac of five days old hens eggs at a dosage of 0 2 ml The eggs were kept at 37° C and candled daily The eggs in which death occurred during the first two days were excluded from further study because such deaths were considered non specific The observation period was ten days after its

extract and 1 2 per cent agar After autoclaving 20 per cent horse serum was added to the agar

In addition to the primary inoculation of eggs direct cultures on PPLO agar and PPLO broth were prepared from the original organ suspension (without antibiotic treatment) From the PPLO broth two consecutive passages were made on the same

substrate at intervals of four days and a culture on a PPLO agar plate was subsequently made from the third subpassage

The PPLO substrates used in addition contained penicillin (100 I U., per ml) and thallium acetate (1 2000) Except for agar the PPLO broth had the same composition as the PPLO agar

The PPLO agar plates were first read after three days incubation and subsequently every day up to six days The PPLO agar plates PPLO broth tubes and blood agar plates were incubated at 37° C.

Inoculation of tissue cultures After 2–4 egg passages the isolated strains were used as undiluted allantoic fluids to inoculate tissue culture tubes (at 0 5 ml per 5 ml) containing monolayers of calf kidney cells The maintenance medium was Hanks balanced salt solution (BSS) containing 0 5 per cent lactalbumin hydrolysate and 5 per cent horse serum

Staining The staining of the isolated strains was done in this study with Giemsa solution diluted 1 20 with a staining period of 2 3 hours (*Klieneberger* 1942)

Serological studies Antisera were produced in rabbits immunized during periods of several months with a 20 per cent dilution (in physiological saline) of the yolk material and allantoic fluid containing the respective strain and using increasing s c and i p dosages up to a considerable amount 20 ml s c and 20 ml i p The sera produced were inactivated in a water bath (30 minutes at 56° C) prior to use

Growth inhibitory tests were carried out by a modification of *Edward & Fitzgerald's* (1954) method Details of the tests will be abvious from the results

Neutralization tests were undertaken in embryonated hens eggs as well as in tissue cultures in these tests the serum was allowed to act on the examined strains for one hour at rom temperature and 18 hours at refrigerator temperature

Agar gel precipitation tests according to *Ouchterlony* (1953) were carried out in Petri dishes into which 20 ml of Nobel agar (Difco) had been poured A metal tube was used to produce in the agar surface reservoirs of 6 mm diameter into which the antigens and antisera were dropped with a Pasteur pipette The reservoirs were spaced at 4 mm The antigens used in the tests were the strains under investigation as undiluted allantoic and tissue culture fluids The antisera too were undiluted The plates were kept one week at room temperature (22° C) and examined daily After the first 24 hours the reservoirs were refilled with the respective antigens and antisera

RESULTS

In the experiments which were started in the autumn of 1963 16 strains have been isolated up to date nine of them from lungs alone and five both from lungs and from nasal mucosa and two from nasal mucosa alone The positive isolations originated from 13 piggeries

The samples obtained from six animals were contaminated by various bacteria and fungi to such a degree that they could not be adapted for egg inoculation with the antibiotics used in the study All these samples had been taken from autopsy material

Properties of the Isolated Strains

The strains all of them primarily isolated in hens eggs were comparatively strongly egg pathogenic The average egg mortality in consecutive passages can be seen from Fig 1

On an average the eggs died six days after the injection range of variation 3–10 days)

Egg Mortality in Consecutive Passages with the Isolated Strains

Egg passage	Average egg mortality %	
I	47	66 %
II	74	
III	55	
IV	75	
V	80	

Fig 1

When the yolk and allantoic fluids of these eggs were cultivated on different substrates growth was obtained on PPLO agar on which a transverse culture of a *Staphylococcus aureus* strain had been established after the preceding culture The growth was most luxuriant in a zone 3–4 mm in width on either side of the staphylococcus stripe decreasing gradually in strength towards the periphery The same growth was also obtained in the surviving eggs in the case of positive isolations The FID_{50} varied between 2 2 and 5 5

None of the strains isolated in the study caused growth on blood agar or on PPLO agar without staphylococci Neither was it possible to achieve growth in PPLO broth directly from the organ suspension but the strains could be brought to multiply weakly in this substrate after some egg passages



Fig 2
Strain N 236 17 on PPLO agar Black area on the right is growth of Staphylococcus aureus 50 ×



Figs. 3 and 4

Fig. 3. Strain No. 24 4 in calf kidney tissue culture seven days after the inoculation. 150 ×

Fig. 4. Outgrowth of normal calf kidney tissue culture. 150 ×

Besides the common growth requirements on cell free media displayed by the isolated strains, the morphology of their growth on such substrates was also fairly consistent. On either side of the staphylococcus stripe small colonies barely visible by the unaided eye were

observed after incubation periods of 3–4 days, which were revealed on microscopic examination as typical mycoplasma colonies (fried egg) Their diameter varied from 0 05 to 0 3 mm (see Fig 2)

Minute coccobacillary bodies typical of mycoplasma were obtained by examination of Giemsa stained smears prepared from the yolk or allantoic fluid as well as from the above described colonies

The strains isolated in the study were also cultivated in calf-kidney tissue cultures, where they within 4–8 days after inoculation produced the cytopathic effect reported as typical of the SEP agent (*Wesslen & Lannek* 1954), which starts as granulation in the cytoplasm, continues as crescent moon appearance of the cells and ends by total cell destruction (see Fig 3 and 4) TID_{50} of the strains varied between 4 5 and 5 5

Serological Investigations

The growth inhibitory tests were carried out by cultivating the strain under investigation as allantoic fluid on a PPLO agar plate, on which subsequently a staphylococcus strain was transversely applied as described Filter paper disks of 1 mm thickness and 10 mm in diameter were then placed on the plate so that they extended across the stap hylococcus stripe Prior to that, each disk had been separately moistened with one of the antisera in respect of which it was desired to examine the strain, while one disk on each plate was moistened with normal rabbit's serum Two drops of the undiluted serum were applied to each disk

The rabbit antiserum produced against one strain (No 115) isolated in the study inhibited the growth both of the homologous strain and of all other isolated strains in an area 3 mm in width around the discs This serum also had the same effect on the growth of the Swedish SEP agent

Antiserum was also produced in rabbit against this known SEP agent from Sweden A growth-inhibiting effect was elicited with this serum against the SEP agent as well as against all strains isolated in the present study

However, these antisera displayed no growth inhibiting effect against the *Mycoplasma granularum* strain obtained from Sweden No growth inhibiting effect of the control disks treated with normal rabbit serum was observed in the present study

With both above-mentioned antisera also qualitative crossneutra lization tests were carried out both in hens' eggs and in tissue cultures Both sera neutralized in 1 4 dilution both their own and the opposite strain at 100 LID_{50} per 0 2 ml in eggs and at 100 TID_{50} per 0 1 ml in tissue cultures They had the same neutralizing effect with respect to the other strains isolated in the present study as well

In the agar gel precipitation tests a strong reaction of the allantoic

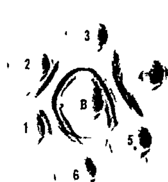


Fig 5



Fig 6

Figs 5 and 6

In the reservoirs No 1 2 4 and 5 allantoic fluids containing the respective strains In the reservoirs No 3 and 6 normal allantoic fluid In the reservoir B antiserum against one isolated strain In the reservoir D antiserum against the Swedish SEP agent

fluid antigens containing the isolated strains as well as the known SEP agent was obtained, both with anti No 115 serum and with anti SEP serum, in the form of six sharp precipitation lines Since the antisera had been prepared by immunizing rabbits with allantoic fluid and no absorption had taken place, it was obvious that some of the lines were due to allantois-antiallantois reaction This was also clearly demonstrated by the normal allantoic fluids employed as negative control antigens Three of the precipitation lines were common both to the antigens containing the strains and to the negative control antigens in their reaction with the above-mentioned antisera The other three precipitation lines were only obtained when allantoic fluids containing strains isolated in the present study as well as the known SEP agent acted as antigens The location of the last mentioned lines was also different in that they were formed considerably closer to the antigen reservoirs and even partly under them (see Figs 5 and 6) No precipitation reaction was seen when the tissue culture fluids of the isolated strains were used as antigens No precipitation reaction was obtained with normal rabbit sera either

DISCUSSION

The mycoplasma strains isolated in the present study can be considered identical with the SEP agent This conclusion is justified considering their analogous growth requirements, egg pathogenicity, typical cytopathic effects in tissue cultures and the serological tests that have been carried out

Eleven out of the sixteen strains included in the study had been isolated *from pigs which were either strongly (seven strains) or slightly*

(four strains) suspect of affection by enzootic pneumonia Five strains had been derived from pigs with mild rhinitis Histopathological examination revealed changes in the lungs suggesting enzootic pneumonia in fourteen out of these sixteen cases

If such samples were excluded as could not be used for the experiment owing to contamination by bacteria or fungi, the SEP agent was isolated in 68 per cent of the animals with suspected enzootic pneumonia

No SEP agent was isolated from animals considered healthy of the basis of preceding examinations, which amounted to 29 per cent of the entire material

Neither did the study reveal any of the other, usually rather more easily isolated mycoplasma species such as *Mycoplasma granularum*, which is fairly common in the respiratory passages of swine in USA (*Ross et al* 1963 *Switzer* 1963)

The facts stated above speak in favour of the opinion that the SEP agent can be considered to represent a specific mycoplasma infection in enzootic pneumonia of swine

A disturbing factor in the work towards a clarification of the aetiology of enzootic pneumonia has been the poor ability of the SEP agent to produce clinical manifestations of the disease under experimental conditions In a later report (*Schulman & Estola* 1965) experimental animal inocultations made with the strains of SEP agent isolated in this study will be described in which distinct histological changes in the respiratory organs of piglets were found

For the diagnosis of enzootic pneumonia, which has proved to be comparatively difficult, the SEP agent isolation technique may be considered a fairly welcome aid However, the success of the investigation involved will depend on fresh samples Autopsy material of the usual quality can rarely be considered a usable and reliable source for the isolation of the SEP agent

SUMMARY

In the present study mycoplasma strains were isolated from cases of swine enzootic pneumonia with the aid of embryonated hens' eggs, PPLO substrates and tissue cultures The strains proved to be identical in morphological and serological examination with the so-called SEP agent originally isolated in Sweden The isolating and identifying technique is described The significance of the SEP agent as an aetiologic factor in swine enzootic pneumonia is discussed

REFERENCES

Bakos K Bjorklund N F Karlsson K A & Dinter Z Procédés de diagnostic de la Pneumonia du porc Bull Off Internat Epiz 58 281-290, 1962

Bakos K & Dinter Z Das SEP Agens Proc XVIIth Int Vet Congr Hannover 1 549 553 1963

Bakos K & Dinter Z Personal communication 1964
Betts A O Respiratory diseases in pigs V *Some clinical and epidemiological aspects of virus pneumonia* of pigs *Vet Rec 64* 283 288 1952
Beveridge W I B & Betts A O Virus pneumonia of pigs Proc XVth Int Vet Congr Stock
Björklund N E
smågrisars s
Dinter Z *Bakos K & Bjorklund N E*
tischen Pneumonie *der* Schweine in der Lunge dieser *Tierart* Mh Tierheilk u 21-30 1954
Dinter Z Wesslén T & Lannek N Die Kultivierung des SFP Agens im Dottersack des Bruteies Zbl Vet Med *4* 581 587 1957
Pneumonie *der* Schweine vom Virus zum
porcine mycoplasma strains
h of PPLO by antibodies J
path bact *68* 23 30
Goodwin R F W & Whittlestone P Experiences *with a schema* for *supervising* pig herds *believed* to be free from enzootic pneumonia (*virus* pneumonia) Vet Rec *72* 1029 1054 1960
Gulrajani T S & Beveridge W I B Studies on *respiratory* diseases of pigs IV Transmission of infectious pneumonia and *its differentiation from* swine influenza *J* comp *Path 61* 118 139 1951
Hjarre A Dinter Z & Bakos K Vergleichende Untersuchungen *uber eine* influenzähnliche Schweinekrankheit *in* Sweden *und* Shopes Schweineinfluenza Nord Vet Med *4* 1025 1045 1952
Hjarre A Uber die enzootische Viruspneumonie beim Schwein Mh Vet Med *12* 503 508 1957
Klieneberger *F* Some new observations bearing on the nature of the pleuropneumonialike organism known as L₁ associated with Streptobacillus moniliformis J Hyg *42* 485-497 1942
Kobe *K* Die *Aetiologie* der *Ferkelgrippe* (enzootischen *Pneumonie* des Ferkels) Zbl Bakt I Orig *129* 161 176 1933
Lannek N & Wesslén T Histological *examinations of tissue cultures* inoculated with a cytopathogenic agent from swine enzootic pneumonia *Acta path et* microbiol scandinav *37* 343 351 1955
Lannek N & Wesslén T Evidence that *the* SEP-agent is *an etiological* factor in enzootic pneumonia *in* swine Nord Vet Med *9* 177 190 1957
L Ecuyer C & Switzer W P Virus pneumonia of pigs Attempts at propagation of the causative agent *in* cell cultures and chicken embryos Can J comp med *vet* Sci 27 91-99 1963
Momberg-Jörgensen H C. Die *Aetiologie* der Ferkelgrippe Berl Munch tierarztl Wschr 54 573 576 1938
Monreal G *Both G & Konz A* Zur Diagnose den enzootischen Pneumonie der
Med *11* 455-468 1964
Obel
Body Rhinitis" des Schweines
Duchl
N *Types of reaction* in c
ol et scandinav 33 231 240
1953
Penttinen K & Rislakki V Om den i Finland förekommande smittosamma grishostans etiologie 2 Adaptionsforsok i embryonerade honsagg och serologiska undersokningar Nord *Vet* Med 5 123 130 1953
Reed L J & *Muench H A* Simple *method* of estimating *50 per* cent end points Am *J* Hyg 27 493 497 1939
Rislakki V Om den i *Finland* förekommande *smittosamma grishostans*
1 Infektionsförsök med försöksgrisar Nord Vet Med *5* 113 124 19
Roberts E D Switzer W *P & L Ecuyer C* Influence of Pasteurella multocida and Mycoplasma *hyorhinis* (PPLO) *on the* histopathology of field cases of swine pneumonia *Cornell* Vet 52 306 327 1962
Ross R F Switzer W P & *Hare C J* Incidence of *certain microorganisms in Iowa* swine Vet Med 58 562 565 1963

Schulman A & Estola T Unpublished data 1965
Shope R F Swine influenza Experimental transmission and pathology J Exptl Med 54 373 385 1931
Slagsvold L Smittsam grisehoste (Parainfluenza) Norsk Vet Tidsskr 54 57 78 158–183 1942
Switzer W P Studies on infectious atrophic rhinitis of swine II Intraperitoneal and intranasal inoculation of young pigs with a filterable agent isolated from nasal mucosa of swine Vet Med 48 392 394 1953
Switzer W P Studies on infectious atrophic rhinitis IV Characterization of a pleuropneumonialike organism isolated from the nasal cavities of swine Am J Vet Res 16 540 544 1955
Switzer W P Report in 6th Nat Pork Indust Conf Madison Wis Nov 14–15 1963
Terpstra J I Het voorkomen van influenza bij varkens in Nederland Tijdsch Diergeneeskd 62 177 186 1935
Takatori I Inaba Y & Omori T Production of floating crystalsoluble in organic solvents (FCSOS) by mycoplasma isolated from swine enzootic pneumonia Nat Inst Anim Hlth Quart 4 151 157 1964
Wesslén T & Lannek N The isolation and cultivation in tissue culture of a cyto pathogenic agent from pigs with enzootic pneumonia (so-called virus pneumonia) Nord Vet Med 6 481 499 1954

Acta path. et microbiol. scandinav. 66: 124–134, 1966

The Department of Toxoplasmosis and Viral Diseases (Head: J. Chr. Siim, M.D.)
Statens Seruminstitut, Copenhagen, Denmark

ISOLATION OF *MYCOPLASMA PNEUMONIAE* (EATON AGENT) FROM PATIENTS WITH PRIMARY ATYPICAL PNEUMONIA

By

K. Lind

Received 20.xii.65

In 1944 *Eaton, Meiklejohn & van Herick* (7) recovered a filterable agent from patients with cold agglutinin associated primary atypical pneumonia, employing chick embryos, cotton rats or hamsters. *Liu*, 1957, (17) adapted the fluorescence technique to the demonstration of the specific antigen in the chick embryos infected with the Eaton agent, and to the titration of antibodies in sera from patients with primary atypical pneumonia. In 1961 *Marmion & Goodburn* (18) suggested that the agent might belong to the genus *Mycoplasma* and this was proved by *Chanock, Hayflick & Barile* (2) who succeeded in growing it on artificial agar medium. The latter was found to be a sensitive medium for the recovery of strains from infected individuals, and furthermore offered a convenient source of antigen for the indirect fluorescent antibody test. Infection of volunteers with the agent resulted in varying degrees of respiratory disease and antibody production (3, 6). In 1962, *Chanock et al.* (4) devised a complement fixation test with a broth cultured, phenol-treated antigen. *Mycoplasma pneumoniae* was proposed as a species name for the Eaton agent (5).

Cold agglutinin positive, primary atypical pneumonia was first described in Denmark by *Siim* (19).

The aim of the present study was to ascertain whether *Mycoplasma*

Grateful thanks are due to the following for permission to study their patients: Professor *Oluf Andersen*, M.D., Dronning Louises Børnehospital; *F. Wi..., Flensborg*, M.D., Department of Pediatrics, Blegdamshospitalet; Professor *H. C. A. Lassen*, M.D., Department of Epidemiological Diseases, Blegdamshospitalet; *F. Neukirch*, M.D., Medical Department T, Rigshospitalet; The late *Folke Tud...*, M.D., Childrens Hospital, Fuglebakken; *J. Vesterdal*, M.D., Department of Pediatrics, Sundby Hospital.

The author is indebted to Mrs. *I. Rasmussen* and Mrs. *A. Schneekloth* for skilled technical assistance.

This study was supported in part by grants from the *P. Carl Petersen Fond* and *The King Christian X Foundation*.

pneumoniae (*M p*) was prevalent in Denmark It was conducted on the basis of the above mentioned methods for cultivation and serological investigation

MATERIAL AND METHODS

Isolation from Patients

77 patients admitted to 6 different hospital departments in Copenhagen in 1962 1963 and 1964 were examined

Throat swabs were taken from patients whose blood specimens were sent to the Department of Toxoplasmosis and Viral Diseases Statens Seruminstitut for cold agglutinin test irrespective of whether this was negative or positive Experience had shown that most of these specimens originated from patients with non bacterial respiratory infections According to the case records on discharge 65 of the patients had suffered from pneumonia and 41 of these were primary atypical or viral pneumonia Three patients had had a respiratory infection without pneumonia while the remaining 9 patients had suffered from various non respiratory diseases

Throat swabs were taken from oropharynx and nasopharynx and immediately spread on agar The agar plates were incubated at 37° C in a moist atmosphere The *Mycoplasma* strains were propagated on agar or in broth They were stored after lyophilization of a broth culture or of a superficial abrasion of agar with colonies suspended in broth

Media The agar medium consisted of seven parts of agar, two parts of non-inactivated and membrane filtered horse serum which had been stored at —70° C for a period from a few days up to several months, and one part of 10 per cent yeast extract The extract was prepared from baker s yeast by boiling for 15 minutes in distilled water and was clarified by decantation and centrifugation at 2000 G for 15 minutes It was then autoclaved and stored at +4° C until used or for a maximum of 3 months The agar was prepared by boiling filtered ox heart infusion broth with 1 per cent Bacto peptone and 0 5 per cent NaCl pH was adjusted to 7 7 by 5 N NaOH and after filtration through paper 1 4 per cent Bacto agar was added This material was then autoclaved and cooled to 48° C and after pre warming to 48° C horse serum and yeast extract were admixed with penicillin 100 units per ml and thallium acetate 1 2000 as final concentrations Aliquots of 30 ml of this medium were poured into plastic dishes measuring 8 5 cm in diameter The broth was prepared from the same constituents without agar, with the addition of 0 5 per cent glucose and phenol red in a final dilution of 2 × 10^{-5} as indicator for acid production

Fluorescent antibody test (FAT) Coons' indirect method was used employing colonies of *M p* grown on agar and subsequently transferred and fixed to slides according to the method of *Clark* as described by *Chanock et al* (3) The following modifications were introduced (a) Fresh guinea pig serum was not used as diluent for the titration of sera sinc
phate buffered saline pH 8
preparations were mounted
phosphate buffered saline I
with fluorescein isothiocyanate (Progressive Laboratories Baltimore, U S A) was absorbed with lyophilized guinea pig liver (11)

A Leitz Ortholux *microscope* was equipped with a HBO 200 watt mercury vapour lamp a BG 12 excitation filter a dark ground oil immersion condenser, an oil immersion fluorite objective 54 × and a 490 nm absorption filter (Leitz) The final magnification was 675 ×

As *test strain* of *M p* the Mac strain isolated by *Eaton* in 1944 was used This strain was sent to Dr *Freundt* at Statens Seruminstitut in 1962 from Dr *Clyde* (Cleveland Ohio U S A) who had propagated it 86 times in chick embryos once in monkey kidney tissue and seven times on agar *Freundt* passed it 9 times on agar before it was employed in this study during which it has been propagated up to 20 times on agar or in broth The Mac strain was used as an antigen for the FAT of all sera In addition the strains isolated were used as antigens for the FAT of sera from the patients concerned

As positive *test sera* from patients with atypical pneumonia, two sera with a high *titre of M p antibodies were used, one of which had kindly been tested by Dr Chanock* (Bethesda, Md, U.S.A.) (CF test) and by Dr. *Hers* (Leiden, Holland) (FAT) in their respective laboratories The negative test sera were negative by the fluorescence technique in dilution 1 20 All sera were heat inactivated at 56° C for 30 minutes and tested for M p antibodies by serial two fold or four fold titrations the first dilution being 1 40 The test was considered positive if the titre was ≧ 160 or a four fold change in titre could be observed in sera from the same patient

Cold agglutinin titrations were performed by a routine method modified by the author (15) *A titre of ≧ 64 or a four-fold change in titre* was considered positive

The *streptococcus MG agglutinin test* was performed according to the method of *Thomas et al* (21), modified only with respect to the titration Part of the serial dilutions made for the cold agglutinin test was used for this test (see 15) The agglutinogen was suspended in saline according to the local standard 8 of density for bacterial suspensions, before addition to each serum dilution A titre of ≧ 16 or a four-fold change in titre of sera from the same patient indicated a positive result

Complement fixation (CF) tests for antibodies against *Influenza A, B* and *C virus Adenovirus* and *Ornithosis virus* were performed by routine methods by the Influenza Virus and the Rickettsia Virus Departments of this institute

The *test for haemolysis* of guinea pig erythrocytes by *M p* according to *Somerson et al* (20) was modified for the purpose of simplification A 10 per cent suspension in phosphate buffered saline pH 7 38 of the washed erythrocytes was poured directly over the agar with the colonies A clear zone of beta haemolysis around each colony of *M p* was seen after 18 hours at 37° C, but not with colonies of other human species of *Mycoplasma*

For comparison four other human species were employed, *viz M hominis* types 1 (PG 21) and 2 (strain Campo)[1], *M salivarium* PG 20 *M fermentans* strain G all provided by Dr *Freundt*, and *M orale* strain Symons made available by Dr *Herderschee*, Amsterdam

Identification of Isolates

The strains isolated were identified as *Mycoplasma pneumoniae* (*M p*) by the following five criteria most commonly used for characterization with respect to genus or species (2 3 8 20)

1 *Morphology*

On the agar as specified above *M p* formed homogeneously granular colonies infiltrating the agar downwards from the centre In contrast to most other human species fried egg forms of colonies were never seen in primary inoculates of *M p* After a few passages on agar the *M p* strains isolated often developed some degree of the fried egg appearance When fully grown the colonies varied from 10 to 150 μ in diameter the size being apparently in*dependent of the density of growth On the contrary* the other human species formed colonies up to from 200 to 500 μ in diameter and the size often seemed to be dependent on the growth density

2 *Growth*

The growth rate of primary isolates was slow The first microscopical demonstration of colonies (usually 10 20 μ in diameter) was not possible until 8 days after inoculation while the other human species were detectable within 2 4 days *M p colonies usually appeared from 10 to 20 days* after primary inoculation

3 *Acid Production*

The ability of *M p* to produce acid in broth containing glucose is only shared by *M fermentans* among the known human species (9) The others (*M hominis* types 1 and 2 *M salivarium* and *M orale*) are base forming (Fig 1)

---

1 Recent investigation by *Lemcke* suggest that strain Campo belongs to the species *M arthritidis* (14)

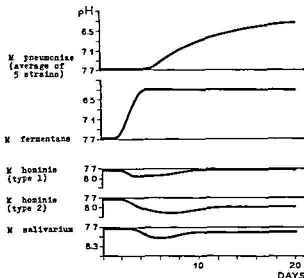


Fig 1

Acid and base formation of different species of *Mycoplasma*
The strains were grown in PPLO broth with 20 per cent horse serum 10 per cent yeast extract penicillin 100 U/ml thallium acetate 1 2000 supplemented with 0 5 per cent glucose and phenol red Ordinate zero = pH 7 7 Each pH unit = 0 3 Curves above zero = acid formation below zero = base formation *M orale* strain Symons which is not shown on this figure was base forming

4 *Haemolysis*

The beta haemolysis of guinea pig erythrocytes around the colonies is another quality characteristic of *M p* as distinct from the other human species

5 *Immunology*

Finally the strains isolated were identified immunologically by the ind rect fluorescence technique

RESULTS

Isolates

The strains isolated could not be distinguished from the Mac strain of *M p* by any of the selected criteria Their immunological resemblance to this strain is shown in Table 1 which also gives a mutual comparison of strains 5 8 and 9 The titres against the Mac strain are compared with the titres against the patients own isolates using one serum with maximum titre from each patient In one of the patients (case No 60) no antibodies against *M p* were demonstrated Good correlation was also found by sera with lower titres

Isolation of M p from Patients

M p was isolated from 14 of 77 patients 53 children and 24 adults (Table 2) Of these 14 isolates 12 originated from a group of 41 patients with primary atypical or viral pneumonia while two were from

antibodies, six with a significant rise in titre, while three had a negative FAT 13 of the 14 patients had a positive cold agglutinin and/or MG agglutinin test Of the remaining 51 patients with pneumonia from whom M p was not isolated, only sera from 49 were examined by the fluorescence technique, 10 of these were positive, two with a rise in titre, and 39 were negative Two thirds of the patients in this group had a positive cold agglutinin and/or MG agglutinin test

TABLE 4

Isolation of Mycoplasma pneumoniae *from 14 Patients with Pneumonia*

No	Sex	Age	Therapy before* culturing from the throat	Throat swab on day after onset of illness
5	♂	5	None	33
8	♂	25	Penicillin	30
9	♂	19	Penicillin	17
17	♀	9	Penicillin + *tetracycline*	17
21	♂	5	*Tetracycline*	25
22	♀	9	None	11
30	♂	9	Penicillin	13
37	♀	9	Penicillin + *tetracycline*	14
38	♂	6	Sulphonamide penicillin + *tetracycline*	16
40	♂	7	Penicillin + *tetracycline*	15
48	♀	14	Sulphonamide + penicillin	25
60	♂	58	Penicillin + *tetracycline*	9
62	♀	6	Penicillin + *tetracycline*	28
72	♀	57	*None*	7
Average				19

* Adequate dosage of the drug for at least three days

TABLE 5

Isolation of Mycoplasma pneumoniae *Serological Data of 77 Patients*

Hospital diagnosis	Isolation of M p	Number of cases	Cold aggl and/or MG aggl positive	Cold aggl and MG aggl negative	FAT for M p antibodies positive	FAT for M p antibodies negative
Pneumonia	Yes	14	13	1	11 (6	3
Pneumonia	No	51*	34	17	10 (2	39
Respiratory infection without pneumonia	No	3	0	3	0	3
No respiratory infection	No	9	5	4	0	9
Total			77		75	

* Sera from two of these patients were not tested by the fluorescent antibody technique Brackets = patients with a significant rise in titre

All the 12 patients without pneumonia had negative tests for *M p* antibodies In this group the three patients with respiratory infection but no pneumonia had negative cold and MG agglutinin tests Of the remaining 9 patients without respiratory infections five had a positive test for cold agglutinins and/or MG agglutinins

TABLE 6

Mycoplasma pneumoniae *Isolation Study*

Patients with Positive Complement Fixation Tests with Influenza Adeno and Ornithosis Virus Antigens

Patient No	Influenza virus C	Adeno virus	Ornithosis virus	Maximum anti *M p* titre	Isolation of *M p*
43	+	—	—	≦40	0
16	—	+		40	0
17	—	+		640	+
20		+		40	0
27	s —	s +		40	0
33	—	f +	s —	≦40	0
39	—	f +		640 (2560)	0
58	—	+		<40	0
59	s —	s +	s —	<40	0
60	—	r +		<40	+
61	—	f +	s —	<40	0
64	s —	s +		<40	0
71	—	+	s —	40	0
51	—	—	+	<40	0
54	—	—	s +	<40	0

Positive complement fixation test = four fold change in titre and/or a titre of ≥ 64 for Influenza and Adeno virus antigens ≥ 120 for Ornithosis virus antigen
r = rise in titre f = fall in titre s = single sera no data

TABLE 7

Mycoplasma pneumoniae *Isolation Study*

Titres of Antibodies against Mycoplasma pneumonia *and Adenovirus in two Patients*

Patient No	Day after onset of illness	Fluorescent antibody test (*M p*)	Complement fixation test (Adenovirus)
17	11	640	32 (64)
	18	160 (640)	128
	25	160 (640)	64
39	13	160 (640)	16
	22	*640 (2560)*	<4

44 children with pneumonia were tested for *M p* antibodies (Table 3) Four out of 25 below 5 years of age had a positive FAT, while this was positive in 14 out of 19 children in the 5–14 year old group

Sera from 75 of the 77 patients were tested by complement fixation

(CF) with Influenza A and B antigen all were negative All but one of the sera from 72 of the patients (64 with paired sera) were negative in the CF test with Influenza C antigen (Table 6) Among 12 out of 75 patients (64 with paired sera) who had a positive CF test with Adenovirus one showed a rise in titre Finally, sera from 18 of the patients were tested for antibodies against Ornithosis virus and two of these had a positive CF test

Out of the 15 patients with positive CF tests for virus antibodies there were two who simultaneously showed a positive test for *M p* antibodies both of these being in the Adenovirus antibody positive group (cases 17 and 39) The serological data concerning these patients as shown in Table 7 indicate that the two patients may have suffered from both an Adenovirus and a *M p* infection The assumption of the latter infection in patient No 17 is supported by the isolation of *M p* on the 17th day of her illness The titres do not permit conclusions to be drawn as to the sequence of the supposed infections

The *test for M p antibodies* was *negative in three of the patients* from whom *M p* was isolated (Table 5) The case histories are briefly as follows

1 *Case No 22* A 9 year old girl was admitted on account of relapsing fever for 1½ months No dullness to percussion or rales could be heard and the roentgenogram showed no infiltrations in the lungs The maximum anti *M p* titre was 80 and there was no change in titre during hospitalization CF tests with Influenza Adeno and Ornithosis virus were all negative The only positive serological reaction was a significant rise in antistreptococcal hyaluronidase titre from 4000 to 20 000 Group A type 12 streptococci were isolated from the throat

2 *Case No 68 In a 58 year old man with bilateral pneumonia no antibodies* against *M p* were demonstrated when sera were tested on the 7th and 36th day of illness The same two sera showed a significant rise in titre of antibodies against Adenovirus while the cold agglutinin titre still remained high (512)

3 *Case No 79* A 57 year old woman who ten years previously had a thoracoplastic operation of the right lung after a tuberculous infection was suffering from right sided pneumonia On the 10th and 16th day of illness her serum showed titres of *M p* antibodies of < 40 and 40 (160) respectively this difference is not significant Unfortunately blood specimens were not taken later Direct microscopy and culture of sputum for tubercle bacilli were negative Cold and MG agglutinin tests as well as CF tests with Influenza Adeno and Ornithosis virus were normal

DISCUSSION

It has not been proved whether the strains isolated were the aetiological agent in the diseases of the patients in this study However evidence has been obtained which suggests that the strains isolated from six of the patients could be of aetiological significance for their pneumonia a significant rise in titre of antibodies against *M p* in the course of the disease having been demonstrated

The limited number of patients and the mode of selection do not *permit conclusions to be drawn as to the incidence of M p* infection

among patients with pneumonia However it is an interesting point that while no *M p* was isolated in the pneumonia group of 27 children below 5 years of age, isolation attempts were successful in 10 of the 19 children in the 5–14 age group with pneumonia *Grayston et al* (10) who isolated *M p* from 42 of 215 pneumonia patients had only one isolation out of 37 in the age group 0–4 years *viz* from a 4 year old child Despite the negative isolation attempts in the present study in the group of children less than 5 years old with pneumonia four out of 25 had a positive test for *M p* antibodies while this was positive for 14 out of 19 children between 5 and 14 years of age In the study of *Jansson et al* (12) 17 out of 56 children from 0 to 5 years old had a positive CF test for antibodies against *M p*, while this test was positive for 49 out of 71 children between 6 and 15 years old All the children were suffering from pneumonia

Five of the nine patients without respiratory infection according to hospital diagnosis had a positive cold agglutinin and/or streptococcus MG agglutinin test Two of these had a titre of 40(160) in the *M p* antibody test Two other patients had

Some of the data presented suggest that patients may suffer from infections due both to *M p* and Adenovirus either concomitantly or in close sequence This is in accordance with the serological data given by *Kingston et al* (13)

The isolation of *M p* from three of the patients with negative tests for *M p* antibodies may be due to persistence of the agent in the pharynges from a present or previously expired mild *M p* infection In the first patient the actual infection was due possibly to streptococci in the second to an Adenovirus in the third patient the cause of infection remains uncertain

It should be pointed out that isolation was possible as late as in the fourth and fifth week of illness Another observation was that half of the patients from whom *M p* was isolated had received tetra cycline in adequate doses for at least three days before the throat swab was taken This is surprising considering the known effect of tetracycline on *M p* infection (13)

SUMMARY AND CONCLUSIONS

Mycoplasma pneumoniae (*M p*) was isolated from 14 out of 77 pa tients admitted to hospitals in Copenhagen *viz* from 10 out of 53 children and from 4 out of 24 adults All isolates originated from a group of 65 patients with pneumonia Isolation attempts in this group were unsuccessful in the 27 children below 5 years of age

Isolation could be accomplished as late as in the fourth and fifth week after onset of illness, even though the patients were treated with tetracycline

REFERENCES

1 *Chanock R M Mufson M A Bloom H H James W D Fox H H & Kingston J R* Eaton agent pneumonia J Amer med Ass 175 213 220 1961
2 *Chanock R M Hayflick L & Barile M F* Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO Proc nat Acad Sci (Wash) 48 41 49 1962
3 *Chanock R M Mufson M A James W D Fox H H Bloom H H & Forsyth B* Recovery of PPLO of atypical pneumonia on artificial agar medium Proc Soc exp Biol (N Y) 110 543 547 1962
4 *Chanock R M James W D Fox H H Turner H C, Mufson M A & Hayflick L* Growth of Eaton PPLO in broth and preparation of complement fixing antigen Proc Soc exp Biol (N Y) 110 884-889 1962
5 *Chanock R M Dienes L, Eaton M D Edward D G ff Freundt E A Hayflick L, Hers, J F Ph Jensen K E Liu C Marmion B P Morton H E Mufson M A Smith P F Somerson N L & Taylor Robinson D* Mycoplasma pneumoniae Proposed Nomenclature for Atypical Pneumonia Organism (Eaton Agent) Science 140 662 1963
6 *Clyde W A Denny F W & Dingle J H* Fluorescent stainable antibodies to the Eaton agent in human primary atypical pneumonia transmission studies J clin Invest 40 1638-47 1961
7 *Eaton M D Meiklejohn M D & van Herick W* Studies on the etiology of primary atypical pneumonia A filterable agent transmissible to cotton rats hamsters and chick embryos J exp Med 79 649 668 1944
8 *Freundt E A* The mycoplasmataceae Morphology biology and taxonomy Munksgaard Copenhagen 1958
9 *Freundt E A* Personal communication 1962
10 *Grayston J T Alexander E R Kenny G E Clarke E R Fremont J C & MacColl W A* *Mycoplasma pneumoniae* infections Clinical and epidemiological studies J Amer med Ass 191 369-374 1965
11 *Hers J F Ph* Fluorescent antibody technique in respiratory viral diseases Amer Rev resp Dis 88 316 333 1963
12 *Jansson E Wager O, Stenström R Klemola E & Forssell P* Studies on Eaton PPLO pneumonia Brit med J I 142 145 1964
13 *Kingston J R Chanock R M Mufson M A Hellman L P James W D Fox H H Manko M A & Boyers J* Eaton agent pneumonia J Amer med Ass 176 118 123 1961
14 *Lemcke Ruth M* The serological differentiation of *Mycoplasma* strains (pleuropneumonia like organisms) from various sources J Hyg (Lond) 62 199-219 1964
15 *Lind K Mansa B & Olesen H* Penicillamine treatment in the cold haemagglutinin syndrome Acta med scand 173 647 660 1963
16 *Lind K* Role of fresh human or guinea pig serum in the fluorescent antibody test for *Mycoplasma pneumoniae* antibodies in human sera Brief report *Acta path et microbiol scandinav 63 639 640* 1965
17 *Liu C* Studies on primary atypical pneumonia I Localization isolation and cultivation of a virus in chick embryos J exp Med 106 455 466 1957
18 *Marmion B P & Goodburn G M* Effect of an organic gold salt on Eaton's primary atypical pneumonia organism and other observations Nature (Lond) 189 247 248 1961
19 *Siim J Chr* 6 cases of atypical pneumonia with increased cold agglutinin titre 2 family endemics Acta path et microbiol scandinav 23 46 476, 1946
20 *Somerson N L Taylor Robinson D & Chanock R M* Haemolysin production as an aid in the identification and quantitation of Eaton agent (*Mycoplasma pneumoniae*) Amer J Hyg 77 122 128 1963
21 *Thomas L Mirick G S Curnen E C Ziegler J E & Horsfall F L* Studies on primary atypical pneumonia II Observations concerning the relationship of a non haemolytic streptococcus to the disease J clin Invest 24 227 240 1945

Acta path et microbiol scandinav 66, 135–142, 1966

Departments of Pathological Anatomy Bacteriology, and Medicine,
Oslo University Hospital, Rikshospitalet Oslo Norway

BACTERIA IN WHIPPLE'S DISEASE

Isolation of a Haemophilus *Strain from the Jejunal Propria*

By

ASMUND KJÆRHEIM, TORE MIDTVEDT, SVERRE SKREDE
and EGIL GJONE

Received 26 vii 65

Whipple's disease, first described in 1907 (22), was for many years thought to be an intestinal lipodystrophy The demonstration of PAS positive material in macrophages of the intestinal mucosa and mesenteric lymph nodes, carried out by *Black Schaffer* in 1949 (1), was considered to lend support to the concept of Whipple's disease as a primary disorder of glycoprotein metabolism The chemistry of the PAS-positive substance in question, however, has not been clarified, neither has its origin been definitely established

During the last years, electron microscopic investigations of peroral intestinal biopsies have shed new light on the aetiology of the small bowel lesions in this disease Several studies have revealed the presence of rod-shaped structures strongly suggestive of small bacteria in the jejunal lamina propria of patients with Whipple's disease (3, 4, 6, 12, 14, 17, 21, 23), and serial biopsies have demonstrated that the bacteria-like bodies disappear during antibiotic treatment (12, 14) The PAS positive granules also tend to disappear, but more slowly than do the bacteria (2, 9, 12, 14, 17) Most authors now seem to agree that microorganisms play a rôle, primary or secondary, in the development of Whipple s disease, and obviously there exists a need for the identification of the "bacillary bodies" regularly found in these patients

Recently, *Caroli et al* (3) isolated a bacterium identified as *Coryne bacterium anaerobicum* from an inguinal lymph node in one patient, and claimed this microorganism to be the cause of Whipple's disease In serial peroral biopsies from the jejunum, *Kok et al* (13) demonstrated *Corynebacteria* and a *Haemophilus* like bacterium in a patient with Whipple's disease, and pointed to *Haemophilus* as the most probable causative agent

The present report deals with the isolation of a *Haemophilus* strain from the intestinal mucosa in a fatal case of Whipple's disease

The authors gratefully acknowledge the advice and criticism of professor *O Torgersen* and the excellent technical assistance of Mrs *Renate Hars*

CASE REPORT

A 46 year old farmer was admitted to the Department of Medicine A the Univer sity Clinic (Rikshospitalet) on June 22 1962 with the chief complaint of diarrhea of 2 years duration

One year previously a generalized lymph node enlargement had been observed Biopsies from cervical and axillary lymph nodes at that time showed nodules of epithelioid cells som of which were surrounded by a few multinuclear giant cells

signs being anaemia and [illegible]
disease was confirmed by
dilated lacteals and nume
The patient was readmi
supportive measures An initial remission occurred but later on he got gradually worse until he died on May 14 1963

Biopsy findings Biopsies were performed simultaneously with 2 Crosby Capsules One of the samples was submitted to bacteriological and virological examination Only bacteria considered as normal in *habitants of the intestine were demonstrated The other biopsy speci* men was divided and fixed in 4 per cent formalin for light microscopy and in cold buffered osmium tetroxide for electron microscopy

Light microscopy The propria of the small bowel was infiltrated by large macrophages (Fig 1) containing PAS positive granules Bacteria were not seen in paraffin sections while examination of 0 5 μ sections from plastic embedded material revealed rod shaped bacteria in the lamina propria barely visible in the light microscope

Electron microscopy The specimen was dehydrated in graded ace tones embedded in Vestopal (19) and contrasted with lead hydroxide according to *Karnovsky* (11) Sections were cut on a LKB Ultrotome and examined in a Siemens Elmiskop I electron microscope

Large amounts of bacteria were seen in the lamina propria located mainly in the tissue spaces just beneath the epithelium (Fig 2) Only a few bacteria were encountered within macrophages The bacteria were about 1 5 μ long and 0 15 μ wide Their cell wall measured ap proximately 150 Å The structural details of the cell wall the plasma membrane the ribosomes and the fibrillar nucleoid material were those generally accepted as typical of bacteria The typical PAS positive

---

Figs 1 2

Fig 1 Light micrograph of jejunal mucosa from patient with Whipple s disease The entire extracellular space of the lamina propria is filled with macr [illegible] °50)

Fig 2 [illegible] Rod shaped bac [illegible] nt to the ep the [illegible] ers of nuclear

TABLE 1

Results of Bacteriological Examination

Origin of samples	Culture media: Blood agar aerobic	Blood agar anaerobic	Chocolate agar	Saboraud medium	Loewenstein medium
Intestinal propria I	*E. coli* *Enterococci*	*E. coli* *Enterococci* *Clostridia*	*Haemophilus* *E. coli* *Enterococci*		—
Intestinal propria II	*E. coli*	*E. coli* *Clostridia*	*Haemophilus* *E. coli*	24° — 37° *E. coli*	—
Intestinal contens	*E. coli*	*E. coli* *Clostridia*	*E. coli*	*Candida albicans* (sparse)	—
Mesenteric lymph nodes	*E. coli* *Enterococci*	*E. coli* *Enterococci*	*E. coli* *Enterococci*	·	—
Retroperitoneal lymph nodes	*E. coli*	*E. coli*			—
Supraenal gland	*E. coli*	*E. coli*	*Haemophilus* *E. coli*	*Candida albicans* (sparse)	—

no growth   not examined

granules in macrophages consisted of a uniform lamellar material In high power electron micrographs this substance could be identified as a double layered material structurally resembling cell membranes and other biological membranes of lipoprotein nature Some granules also contained dense slender rods of the same dimensions as bacteria but otherwise lacking their morphologic characteristics Most identifiable bacteria in macrophages were located in vacuoles outside the granules

Autopsy findings Gross and microscopical examinations of all parenchymal organs were performed The jejunum was moderately widened with a thickened mucosa The mesenteric lymph nodes were slightly enlarged and of firm consistence Retroperitoneally several enlarged firm lymph nodes were found A fibrous pericarditis and a left sided pleural effusion were demonstrated

Microscopically the jejunal submucosa contained small rod shaped bacteria like bodies with a predominantly extracellular location and numerous large macrophages with PAS-positive granules The mesenteric and retroperitoneal lymph nodes contained large numbers of macrophages with PAS-positive granules and large extracellular aggregates of a slightly PAS-positive substance This substance could also be stained with Congo red and methyl violet but not with Sudan red Hyaline deposits with the staining properties of amyloid were also found in the renal glomeruli A more detailed description of this finding has been given elsewhere (20) Tuberculous lesions were not demonstrated There were no signs of septicaemia

Bacteriological studies At autopsy special precautions were taken to avoid contamination of the bacteriological samples After opening of the intestine the intestinal mucosal surface was irrigated with sterile physiological saline The mucosa was incised the edges were burned off and the mucosa was carefully dissected from the submucosa Samples were taken from the submucosal tissue fluid in two different areas of the intestine. Material from lymph nodes was ground in a sterile mortar before inoculation

Primary cultures were made on different conventional culture media Incubations were made both aerobically and anaerobically at 24° C and 37 C In Table 1 some of the results of these investigations are recorded In addition incubations were also made on lactose agar tryptose agar Brain Liver Heart semisolid medium (Difco) Tetrathionate broth (Difco) and dextrose broth The results are listed in Table 1

The aerobic plates were examined daily and subcultures were made from each type of colony The anaerobic plates were examined in a similar way after 72 hours of growth and aerobic as well as anaerobic subcultures were made Material from the fluid media was taken after 24 and 48 hours of cultivation and subcultured both in fluid media and blood agar plates for further isolations The isolated strains were identified by morphological and biochemical criteria when pure cultures had been achieved Inoculation of 0 5 ml of a 10 per cent saline extract

8 *England M T French J M & Rawson A B* Antibiotic control of diarrhea in Whipple s disease A six year follow up of a patient diagnosed by jejunal biopsy Gastroenterology 39 219 233 1960
9 *Hargrove M D Jr Verner J V Smith A G Horswell R R & Ruffin J M* Whipple s disease Report of two cases with intestinal biopsy before and after treatment Gastroenterology 39 619–624 1960
10 *Hollenberg M* Whipple s disease a case report with enzyme histochemical and electron microscopic findings Am J Med 32 448–459 1962
11 *Karnovsky M J* Simple methods for staining with lead at high pH in electron microscopy J Biophys Biochem Cytol *11* 729 732 1961
12 *Kent T H Layton J M Clifton J A & Schedl H P* Whipple s disease Light and electron microscopic studies combined with clinical studies suggesting an infective nature Lab Invest *12* 1163 1178 1963
13 *Kok N Dybkær R & Rostgaard J* Bacteria in Whipple s disease I Results of cultivation from repeated jejunal biopsies prior to during and after effective antibiotic treatment Acta path et microbiol scandinav *60* 431 449 1964
14 *Kurtz S M Davis T D Jr & Ruffin J M* Light and electron microscopic studies of Whipple s disease Lab Invest *11* 653 665 1962
15 *Omland T* Serological studies on *Haemophilus Influenzae* and related species Examination of type specific antigens of *H Influenzae* by means of the gel precipitation method Acta path et microbiol scandinav 59 507 590 *1963*
16 *Pittman M* In Bergey s Manual of Determinative Bacteriology 7th edition p 406 Ed by Breed R S Murray E G D & Smith N R The Williams & Wilkins Company Baltimore 1957
17 *Rostgaard J* Bacteria in Whipple s disease 2 Combined electron and light microscopy Acta path et microbiol scandinav *60* 450 461 1964
18 New York 1962
19 ultramicrotomie
20 path et micro
21 s disease in the
(*Conference on*
Houwink A L
croscope Delft
Netherlands 1960
22 *Whipple G H* A hitherto undescribed disease characterized anatomically by
r c lymphatic
23 microscopy in
the intestine

Acta path et microbiol scandinav 66 143–144 1966

BRIEF REPORT

REVERSE VARIATIONS BETWEEN *CANDIDA ALBICANS* AND *CANDIDA TROPICALIS*?

By *J Brown Thomsen*

In 1939 *Cavallero* studied the dissociation in one-cell cultures of *Mycotorula albicans* (*Candida albicans*) In two cases he found variants which were different from the original culture In the first case it was a variation in the micromorphology of the yeast while the second showed variation in the ability to ferment sucrose

The latter variant fermented sucrose fairly rapidly but nevertheless *Diddens & Lodder* (1942) regarded it as belonging to the species *Candida albicans* in view of the fact that many of their strains could bring about a very slow fermentation of sucrose They thus considered the sucrose fermenting ability of Cavallero s strain not as a qualitative but as a quantitative difference

In some experiments I have found variations in one-cell cultures of *Candida albicans* classified according to *Lodder & Kreger van Rij* (1952) The original cultures produced chlamydospores in rice agar and the filamentation reactions (*Taschdjian et al* 1960 *Stenderup & Brown Thomsen* 1964) were positive after 2 hours at 35° C in human serum The fermentation and assimilation reactions were in accordance with the description of the species *Candida albicans*

Using macro and micromorphological criteria for the detection of variants three variants (1A 1B and 2A) were found in two one cell cultures (1 and 2) Two of the variants (1A and 1B) occurred spontaneously on malt extract agar and the third (2A) was found on malt extract agar after preservation for 5 months in sterile water

Some of the characteristics of the original one-cell cultures and of three variants are shown below

Strain No	1	1A	1B	2	2A
Chlamydospore	+	—	—	+	—
Filamentation	+	—	—	+	—
Fermentation					
Glucose	+	+	+	+	+
Galactose	+	+	+	+	+
Sucrose	—	+	+	—	+
Maltose	+	+	+	+	+
Lactose	—		—	—	—
Assimilation					
Beta glucosides	—	+	+	—	+
Arbutin splitting	—	+	+	—	+

The [illegible]

Although it was less extensively studied a variant which fits in with the characteristics of Candida albicans have been isolated from a one-cell culture of a Candida tropicalis strain

Received 10 1 66 *from the Institute of Bacteriology University of Aarhus* (*Head A Stenderup*)
Supported by *Statens almindelige Videnskabsfond*

The observations just mentioned raise the question whether a distinction between Candida albicans and Candida tropicalis as two species can be maintained also taking into consideration the very close antigenic relationship between the two (*Tsuchia et al* 1956 *Hasenclever et al* 1961)

Further experiments will be reported later

References 1 *Cavallero G* Mycopathologia 1 4 227–265 1938/39 —2 *Diddens H A & Lodder J* Die anascosporogenen Hefen Zweite Halfte p 45 1942 —3 *Hasenclever H F Mitchell W & Loewe J* J Bact 82 574 577 1961 —4 *Lodder J & Kreger van Rij N J W* The Yeasts A taxonomic study p 471 1952 —5 *Stenderup A & Brown Thomsen J* Acta path et microbiol scandinav 62 303 304 1964 —6 *Taschdjian Claire L Burchall M A & Kozinn P J* A M A J Dis of Children 99 212 215 1960 —7 *Tsuchia T Kamijo K Fukazawa Y Miyasaki F & Kawakita S* Yokohama med bulletin 7 127 132 1956

Acta path. et microbiol. scand. 66: 145–153, 1966

The Institute of General Zoology, University of Copenhagen, Dental Department, University Hospital, Copenhagen, and Department of Oral Pathology, Royal Dental College, Copenhagen

AN ELECTRONMICROSCOPIC STUDY OF ORAL MUCOSAL LESIONS IN ERYTHEMA MULTIFORME EXUDATIVUM

Report of a Case

By

F. A. von Bülow, E. Hjørting-Hansen and M. Ulmansky

Received 4.xi.65

Numerous reports have dealt with the clinical picture of erythema multiforme exudativum (e. m. e.), but very few have described the histopathological changes of the oral mucosa.

PREVIOUS INVESTIGATIONS

Cooke 1960 reported an eosinophilic degeneration of the prickle cells in the oral mucosa as a frequent but not pathognomonic finding in patients with e. m. e. *Pierard & Whimster* 1961 and *MacVicar et al.* 1963 described the light microscopic findings of the cutaneous manifestations of e. m. e. as being characterized by spongiosis, transepidermal exudation and occasionally liquefaction degeneration in the epidermis, and by perivascular infiltrations mainly of lymphocytes in lamina propria, subepidermal bulla formation and rather extensive peribullous lymphocytic infiltration in the connective tissue papillae. *MacVicar et al.* found that the basement membrane was attached to the roof of the bulla. In their electronmicroscopic study *Caulfield & Wilgram* 1962 found an inter- and intracellular oedema in the epithelium, a reduction in the number of the desmosomes at the base of some of the basal cells. The basement membrane showed thinning and sometimes disruption. The changes in the connective tissue were found to consist of a swelling of collagen fibers and a deposition of plasma proteins between the individual fibers.

As no electronmicroscopic study has been done on oral mucosa lesioned by e. m. e., the aim of the present study is to report the findings of a single case.

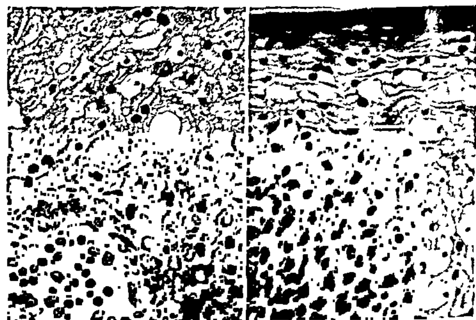
Requests for reprint should be addressed to Dr. E. Hjørting-Hansen, Department of Oral Pathology, 4 Universitetsparken, Copenhagen Ø.

CASE REPORT

The patient was a 24-year old women, who during the past 5 years has had numerous attacks of e m e with typical skin and oral mucosa lesions The actual attack started 5 days earlier with skin affections, and 2 days before the biopsy was taken the patient noticed changes in the oral mucous membrane On examination tiny erosions were seen in the buccal mucosa In the lower lip changes suggesting intra-epithelial liquid accumulation were seen in several areas, each of these was of pin head size The mucous membrane was slightly erythematous In local infiltration analgesia excisional biopsies were made of these early lesions Care was taken to place the analgeticum as far away as possible from the biopsy-area The tissue for light microscopy was fixed in neutral formalin and stained with haematoxylin eosin and with periodic acid-Schiff (PAS) before and after treatment with diastase The tissue for electronmicroscopy was immediately fixed in OsO_4, embedded in Vestopal W, sectioned on LKB untramicrotome, contrasted with uranyl acetate and examined in a Siemens Elmiscop I One micron thick sections for orientation were stained with toluoidine-blue-pyronine, (*Ito & Winchester* 1963), Fig 1 Smears for exfoliative cytology and herpes simplex virus cultures were taken from similar adjacent oral lesions

Light Microscopic Examination

The cells in the surface layer were flattened and eosinophilic The spinous cell layer demonstrated two forms of oedema, a marked intercellular oedema and an intracellular oedema forming vacuoles Both types of oedema extended almost to the surface, Fig 2. Intracellular accumulation of PAS-positive material was found in the spinous layer, and this material was not removed by diastase-treatment. No PAS-



Figs 1–2

Fig 1 Orientation section for electronmicroscopy Toluidine-blue-pyronine × 585
Fig 2 Section for light microscopy Note the oedematous changes and the presence of lymphocytes within the epithelium Haematoxylin-Eosin × 390

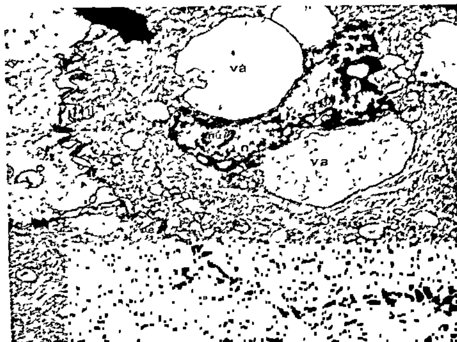


Fig 3

A heavily vacuolated cell in the spinous layer Two large vacuoles (va) are seen on each side of the nucleus (n) The nuclear membrane has disappeared There is a clumping of the chromatin, whereas the nucleolus (nu) is still present The cytoplasm is filled up with vacuoles, the organelles have disappeared, only the fine filaments persist The junction with other cells shows several desmosomes (de) There is no intercellular oedema × 19000 The bar represents 1 micron

positive material was demonstrated in the vacuoles In the basal layers of the epithelium lymphocytes were seen between tht epithelial cells Because of the heavy inflammation it was not possible to distinguish the basement membrane, neither in sections stained with haematoxylin-eosin nor in sections stained with PAS In the juxtaepithelial layers the inflammation was more diffuse and intense and a marked oedema was present In the submucosa perivascular infiltration with lymphocytes and a few plasma cells were found.

Electron Microscopy

The most superficial cells of the epithelium had the same appearance as in normal epithelium The superficial part of the stratum spinosum showed signs of oedema that increased to a marked oedema in the rest of the stratum spinosum and in the basal layer In the stratum spinosum the oedematous changes were of two types:

1 In areas where the epithelial cells were heavily vacuolated, the intercellular space was nearly normal, i e very little oedema was found

Fig 4

Fig 5



Fig 6

The dermo epidermal junction The basement membrane can be followed from the left of the picture via the bottom of the picture to the upper edge in the right side A rounded lymphocyte is seen between the epithelial cells The epithelial cell to the left contains a small but distinct Gogi zone (gz) × 17500

The cells showed no organelles in the cytoplasm, whereas tonofilaments remained The nuclei showed an alteration in chromatin pattern, either as a peripheral clumping, Fig 3, or as a more or less pronounced disintegration, Fig 4, with a disappearance of the nuclear membrane

2 In areas with marked intercellular oedema, the single cells demonstrated mitochondriae, a Golgi zone, numerous ribosomes and a nuclei, that had a normal appearance, Fig 5 Some cells contained a few small vacuoles Yet the surface of these cells was very irregular with a stretching of the desmosomal areas

The electron density of the material deposited between the cells was

---

Fig 4 Another type of intracellular oedema, showing vacuoles (va) around the nucleus (n), the nuclear membrane has disappeared The chromatin shows a disintegration The cytoplasm contains several vacuoles, the organelles have disappeared only the fine filaments are left There is no intercellular oedema × 19000

Fig 5 A cell in the spinous layer The space between the cell and the neighbouring cells is enlarged, leading to a stretching of the intercellular junctions The cell contains several mitochondriae (m) and a Golgi zone (gz) The cell and the neighbouring cells have a few, small vacuoles × 31000

Fig 7

Fig 8



Fig 9

Capillary in the connective tissue with endothelial cells (en) lymphocyte (l) erythrocytes (e) and plasmacells (pl) The capillary is somewhat contracted possibly due to the local anaesthesia The junction between the endothelial cells shows a thickening of the cell membrane (arrow) The basement membrane around the capillary is unbroken × 15500

the same as for the intracellular material found in the vacuoles in both types of oedema Lymphocytes were frequently seen between the cells of the stratum spinosum and the basal layer The basal cells demonstrated many small mitochondriae with typical polar arrangement Furthermore the cytoplasm contained numerous ribosomes a Golgi zone and a large nucleus The attachment to neighbouring epithelial cells consisted of desmosomes Fig 6 Towards the basement membrane the epithelial cells showed a thickening of the cell membrane much like a desmosome The basement membrane could be followed unbroken in all sections, *Fig 6 At some occasions a cell could be seen* to pervade the basement membrane on its way from the connective

---

Fig 7 The passage of a cell possibly a lymphocyte through the basement membrane into the oedematous space between the basal cells (bc) There are no granules or organelles in the pseudopodium Notice that the basement membrane is in contact with the passing cell (arrow) × 23000

Fig 8 Part of fibroblast showing normal mitochondria nuclear membrane and collagen fibers leaving the cell × 35200

tissue into the epithelium, Fig 7 In the connective tissue the fibroblasts were apparently normal, producing normal collagen fibres, Fig 8 The fine filaments connecting the basement membrane with the collagen fibers were normal in size and number The capillaries showed no changes, especially no separation between the endothelial cells, Fig 9

The slides for exfoliative cytology were stained after Papanicolaou's method No changes were found indicating a viral infection Neither in cultures nor by inoculation in mice could herpes virus be demonstrated

DISCUSSION

The light microscopic findings in this reports are nearly all in agreement with those described in the previous reports concerning skin It was impossible to locate the basement membrane because of the inflammatory changes The eosinophilic degeneration within the spinous cell layer described by *Cooke*, 1960, and also seen by us, probably corresponds to the PAS positive areas found in the present investigation It is interesting to note that the intracellular vacuoles did not demonstrate PAS-positive material, but, according to the electron micrographs, contained intercellular fluid The PAS-positive material was located around the vacuolated areas within the cells and never between the cells The findings by *Caulfield & Wilgram*, 1962 could not be confirmed, as it was found that the space between the endothelial cells of the capillaries were closed by terminal bars, and that the basement membrane surrounding the capillaries was unbroken

The oedematous changes in the stratum spinosum were either intracellular or intercellular Nuclear changes were only found in the cells with intracellular oedema Considering the pathogenesis of these lesions one might speculate, that the intercellular oedema is primary since in these areas other cytological details are normal except for the stretching of the desmosomal areas The intracellular changes may follow with disappearance of the nuclear membrane, mitochondria and other organelles This may lead to complete disruption of single cells and may contribute to the bulla formation

Newman, 1956, demonstrated in the electron microscope viral type bodies in filtrates of saline washings from the mouth of patients with e m e *Schmidt*, 1961, was able to isolate herpes simplex virus from an aspirate from a blister on the genitals of a patient with e m e In the present study no signs of viral infection were found, neither in the cytological changes nor in cultures of aspirates from vesicles

SUMMARY

The electronmicroscopical findings in a case of erythema multiforme exudativum are reported Two types of oedematous changes were found

in the spinous cell layer of the epithelium, an intercellular and an intracellular oedema No changes were found in the connective tissue except for oedema, and infiltration with lymphocytes and plasma cells There were no signs of viral infection

REFERENCES

Caulfield J B & Wilgram G F An electron microscopic study of blister formation in erythema multiforme J invest Derm *39* 307–316 1962

Cooke B E D The diagnosis of bullous lesions affecting the oral mucosa Brit dent J *109* 131 138 1960

Ito S & Winchester R J The fine structure of the gastric mucosa in the bat J Cell Biol *16* 541 577 1963

MacVicar D N Graham J H & Burgeon S F Dermatitis herpetiformis and bullous pemphigoid a comparative histopathological and histochemical study J invest Derm *41* 289 300 1963

Newman C W Electron microscope study of erythema multiforme Oral Surg *9* 962 969 1956

Piérard J & Whimster I The histological diagnosis of dermatitis herpetiformis bullous pemphigoid and erythema multiforme Brit J Dermat *73* 253 266 1961

Schmidt H Isolation of herpes simplex virus from blisters of a patient with Steven Johnson s Syndrome Acta derm venereol (Stockh) *41* 53 55 1961

Acta path et microbiol scandinav 66 154–168 1966

The Rheumatic Research Laboratory, University Institute of Pathological Anatomy (Head Professor G Teilum M D) and Department of Medicine Sundby Hospital (Former Head Professor K Iversen M D), Copenhagen Denmark

SYSTEMIC (PRIMARY) AMYLOIDOSIS ASSOCIATED WITH AN IgM (β_2M) PARAPROTEINAEMIA

Biochemical, Histochemical and Immunohistochemical Investigations

By

P RANLOV and P ELLING NIELSEN

Received 31 viii 65

Very few cases of primary macroglobulinaemia (*Waldenstrom*) associated with amyloidosis have been reported In the majority of these cases the amyloid deposits seem to have been localized as in the "secondary" type of amyloid disease (*Vandema* 1954, *Braunsteiner et al* 1956, *Jannes et al* 1961) In a single report the localization was quite unusual, that is, to the peripheral nerves and their vessels (*Nick et al* 1963)

"Secondary" or "symptomatic" macroglobulinaemia without the features characteristic of Waldenstrom's disease purpura lymph adenopathy, and infiltration of the bone marrow and other tissues with 'lymphocytoid' cells is by no means a rare condition It can be seen in connection with hepatic cirrhosis, nephrosis, rheumatoid arthritis eosinophilic granuloma (*Scheiffarth & Gotz* 1960, *Fessel* 1962 *Golhoni et al* 1965), carcinoma (*Laurell et al* 1957), lymphosarcoma (*Onal & Cooper* 1960), chronic lymphocytic leukaemia (*Seligman et al* 1959), and congenital syphilis (*Willi et al* 1954) *Scheiffarth & Gotz* (1960) mention that "secondary" macroglobulin has more antigenic determinants in common with the normally occurring macroglobulin than is the case in Waldenstrom s disease

Clausen & Christensen (1964) were the first to report the association of primary amyloidosis with paraproteinaemia In each of their four cases a gamma SS (IgG) paraprotein was found In addition one of their patients disclosed a gamma A (IgA) paraprotein The possibility of myeloma could be excluded in all their cases

A 'secondary' or 'symptomatic' macroglobulinaemia accompanying a "primary" amyloidosis is not seen to have been reported in the liter

These Investigations have been supported by *The Danish League Aginst Rheumatism*

ature Therefore, in the present paper, a case of systemic (primary) amyloidosis associated with an IgM paraproteinaemia will be described in detail together with an account of some relevant biochemical, histochemical and immunohistochemical investigations

CASE HISTORY

(Sundby Hospital rec 2211/62) E V M a 71 year old male retired schoolteacher

Until the onset of the cardiac symptoms his health had always been perfect

First admission November 8 1962 he was admitted with an abscess in the nasal septum In the months preceding admission he had suffered fatigue and increasing loss of weight Because of the uraemia he was transferred to the medical department where he was found in uraemic coma with a severe acidosis the actual pH of the capillary blood being 7 1o2 with a standard bicarbonate 9 1 mEq/l (Astrup method) In a couple of days he was rehydrated and during the rest of the five weeks stay in hospital his general condition improved considerably following treatment with blood transfusions anabolic steroids and sodium bicarbonate The aetiology of the renal disease remained obscure and a kidney biopsy was unsuccessful

Laboratory investigations Haemoglobin 11 8 g/100 ml E S R 125 mm/h MCV 104 MCHC 28 colour index 0 93 thrombocytes 415 000 cmm Coagulation tests normal Serum alkaline phosphatases 15 12 mMol/l/h GO transaminases 1 97 mMol/l/h thymol turbidity 0 53 bromosulphalein retention test 17 per cent Serum electrolytes and acid base status normalized The proteinuria oscillated between 1 5 and ... mg 100 ml Blood urea

e

ied band in the gamma

2 57 α_1 globulin 0 32

0 ml In the *immuno electrophoresis* (dr *J Clausen* Institute of Biochemistry University of Copenhagen) a picture similar to that of Waldenstrom s disease was found after depolymerization with cysteine

After discharge from hospital h s general condition again deteriorated He de

le was

ps and could not be felt The heart was enlarged and the pulse arrhythmic The liver was firm and could be felt 5 fingers below the costal margin There was considerable ascites and severe oedema symmetrically on both crura and feet

This patient s main problem was his severe heart failure for which reason intensive therapy with diuretics digitalis and sodium bicarbonate was initiated In spite of this he steadily deteriorated developed hydrothorax and succumbed in heart failure after 4 weeks in hospital

Laboratory investigations Haemoglobin 12 7 g/100 ml E S R 123 mm/h MCV 114 MCHC 29 colour index 1 03 thrombocytes 466 000 cmm Coagulation tests normal

lesions

Total serum protein was 7 15 g/100 ml Repeated *paper electrophoresis* of serum showed constantly the same sharply defined abnormal band in the γ area of the strip Albumin 2 92 α_1 globulin 0 34 α globulin 0 49 β globulin 0 89 and the γ area 2 58 g/100 ml

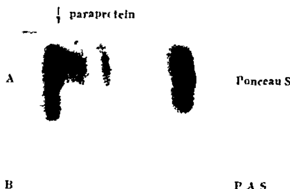


Fig 1
Cellulose acetate electrophoresis of serum A Ponceau S stain The abnormal band can be seen in the gamma fraction B the paraprotein is seen to be strongly P A S positive indicating a high glycoprotein content (normal gamma fractions are P A S negative)

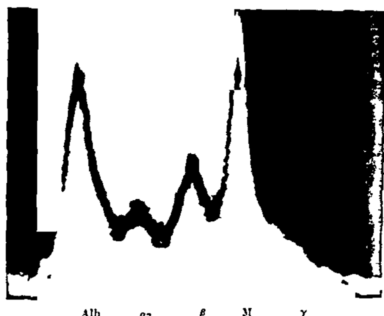
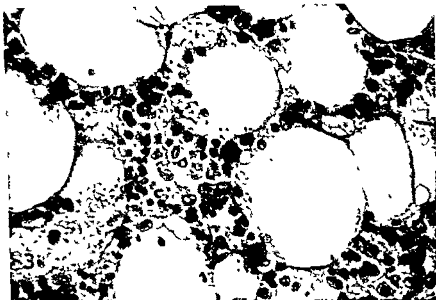
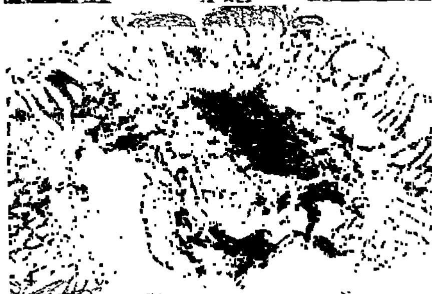


Fig 2
Tiselius electrophoresis of serum showing the abnormal peak in the gamma area (see text)

Serum electrophoresis performed in *cellulose acetate* P A S stained for glycoproteins showed a hight carbohydrate content in the abnormal band in contrast to normal IgG (Fig 1)

Tiselius electrophoresis (dr A *Harboe* The Protein Laboratory University of Copenhagen revealed 2 gamma fractions One slower corresponding to normal





Figs 3–4

Fig 3 Section of sternal marrow No abnormal cells or inclusions are seen P A S stain × 560

Fig 4 Biopsy of rectum Amyloid deposits in submucosal vessels van Gieson Hansen stain × 35

gamma (IgG) constituting 11 per cent of the total serum protein or 0 75 g/100 ml (subnorn
the total
fraction
molecula

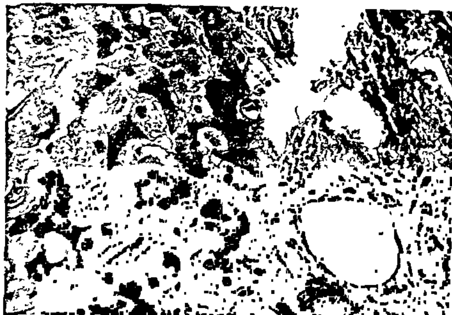


Fig 5

Liver biopsy showing excessive amyloidosis of the sinusoidal walls causing compression of the parenchymal cells P A S stain × 350

With serum, urine and spinal fluid *immuno electrophoresis* was repeated (dr *J Clausen* Institute of Biochemistry, University of Copenhagen) This time IgG and IgA were found to have decreased Again the amount of IgM was found to have raised in the fastest moving parts of its line of precipitation

Sternal Marrow Biopsy

Differential count showed a normal distribution Plasma cells contributed only with 1 per cent, lymphocytes with 4 per cent, and monocytes with 1 per cent of the total count The bone marrow was slightly hyperplastic but no abnormal cells could be found P A S staining revealed nothing abnormal, especially no inclusions (Fig 3)

Biopsy of the Rectum

The submucosal vessels exhibited subendothelial deposits of an amorphous material, most pronounced in the larger and medium sized arteries The material showed weak metachromasia with toluidine blue and was weakly Congo-positive It yielded strong fluorescence in ultra-violet light after treatment with Thioflavine T (Fig 4)

Liver Biopsy

Throughout the liver tissue was abundant amorphous material The deposits were mainly localized to the sinusoidal walls, thus causing compression of the parenchymal cells The morphology was that of amyloid (Fig 5) The material stained metachromatically with methyl violet and toluidine blue It was moderately Congo-positive and gave marked fluorescence with Thioflavine T

AUTOPSY

The immediate cause of death turned out to be cardiac decompensation and bronchopneumonia



Fig 6
Section of left kidney with tumour

Central and peripheral lymph nodes were of normal size and of soft consistency The heart weighed 610 g both halves were dilated and the walls showed hypertrophy and stiffness The aortic valves were rather stiff moderate atheromatosis was found in the coronary arteries but signs of recent or earlier infarctions could not be detected In the lungs were small atelectases they were rather heavy and scattered broncho pneumonias were found The thymus was absent

Abdomen contained 300 ml of ascites The liver was enlarged 31 × 21 × 12 cm with a smooth surface Its stiffness was marked The cut surface was waxy or greasy The gall bladder and ductus choledochus were both normal without stones or strictures Also the spleen was enlarged 13 × 8 × 6 cm weighing 265 g It was very firm and the cut surface was waxy The kidneys were small finely granulated 9 × 5 × 3 cm Their cortex was found narrowed All papillas were intact A small less than 1 cm superficial tumour was found in the middle of the anterior surface of the left kidney It was round well defined and did

not stick to the renal capsule (Fig 6) The rest of the abdominal contents showed nothing of interest

The brain, the cerebral vessels, and the meninges were all macroscopically normal So was the bone marrow

MICROSCOPY

Sections of paraffin embedded tissues from the following organs were studied the lungs, heart, thyroid kidneys, adrenals prostate, liver, spleen, lymph nodes, bowel, intestines, skin, voluntary muscles, bone marrow and brain

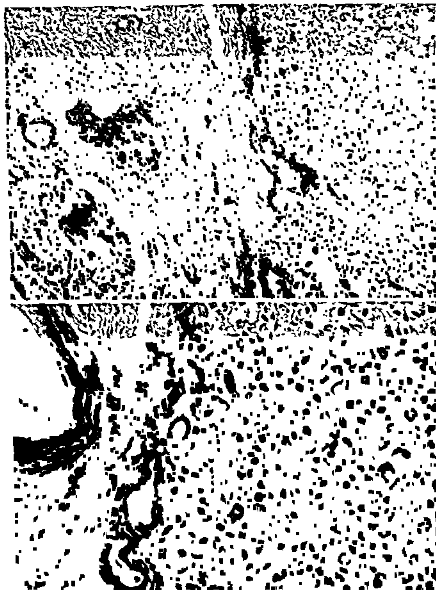
The lungs exhibited the earlier described amorphous deposits in the vessels, but in addition the same material could be recognized in the walls of single alveoli Also the small vessels in *the heart* showed amyloid changes besides some interstitial infiltration in the myocardium In *the kidneys* most glomeruli were found to be stuffed with amyloid material which also surrounded many tubules (Fig 7) The structure of *the adrenals* was blurred because of heavy amyloid infiltrations The section of *liver* tissue showed changes identical with those described in the biopsy specimen (Fig 5) In *the spleen* only sparse remnants of the original pulp were seen scattered between the amorphous deposits In *the lymph nodes* only a few germinal centres could be recognized between the homogenous masses In every single organ, the bone marrow inclusive, the central nervous system being the sole exception, the same characteristic deposits were found in the walls of the smaller and medium sized vessels In all the sections studied this substance was weakly metachromatic with methyl violet and slightly positive with Congo red It stained positive with Alcian blue, most pronounced in the periphery of the deposits There was a strong fluorescence with Thioflavine T

The kidney tumour was seen surrounded by a fibrous capsule. The tumourous tissue was composed of an uncharacteristic network of rather pale small cells Larger cells with a greyish cytoplasm were seen interposed together with a pale nucleus with irregular chromatine The picture was far from typical Mainly because of its small size (less than one centimeter) a diagnosis of renal cortical adenoma, possibly carcinoma, was suggested (Figs 7–8)

IMMUNOHISTOCHEMICAL INVESTIGATIONS

The tissues used were biopsy specimens from the liver bone marrow and rectum In addition spleen tissue recovered less than ½ hour after death was used Immediately following removal the specimens were frozen down to —70° C and kept stored at this temperature until the investigation Sections were cut in a cryostat 5 microns thick followed by incubation with a conjugated antiserum for 30 minutes Finally they were washed in Coons phosphate buffer

Four antisera were employed a rabbit antiserum against human Cohn's fraction II, prepared in this laboratory after repeated intramuscular injections of pooled



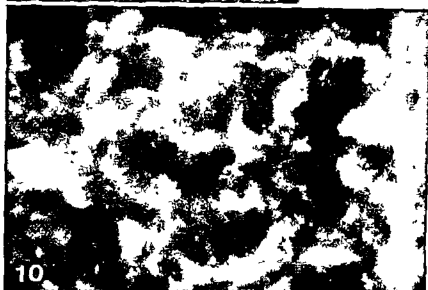
Figs 7–8

Fig 7 Same section as Fig 6 To the left amyloid deposits in glomeruli, tubules *and renal vessels To the right renal tumour (renal cortical adenoma possibly carcinoma)* The tumour is seen surrounded by a capsule Alcian blue stain × 140

Fig 8 Same section as Figs 6 and 7 Alcian blue stain × 350

purified human globulin into albino rabbits The antiserum was tested immuno electrophoretically Three commercial rabbit antisera against purified human IgG IgA and IgM, respectively (Obtained from Centraal Laboratorium van de Blood *transfusiedienst van het Nederlandsche Roode Kruis, Amsterdam, Holland)* The specificities of these three sera were tested immunoelectrophoretically These four





Figs 9–10

Fig 9 Phase contrast photomicrograph of section of liver biopsy specimen showing amyloid deposits between liver cells × 400

Fig 10 Fluorescence photomicrograph of exactly the same section as Fig 9 in

human IgG IgA and IgM respectively UV light × 400

antisera were conjugated with fluorescein isothiocyanate (0 025 mg/mg protein) as stated by *Coons & Kaplan (1950)* All conjugates were dialysed against saline and absorbed with powdered acetone dried guinea-pig liver They were kept at −20° C. A Leitz Ortholux microscope was used with equipment allowing simultaneous fluorescence and phase contrast microscopy through a light-field condensor It

was connected with a high pressure mercury lamp (Osram HBO 200) behind an LG 1 exciter filter and a colourless UV-absorbing secondary filter

RESULTS

Fluorescence of the amyloid material was not observed in any of the sections from spleen, liver, bone marrow or colon regardless of the type of antiserum used This is demonstrated in Fig 10, which shows weak fluorescence of the liver parenchyma cells following incubation with antiserum against Cohn's fraction II, while the interjacent amyloid deposits are seen completely devoid of fluorescence Exactly the same section is shown in Fig 9, this time in phase-contrast The amyloid material is seen to correspond closely to the dark areas in Fig 10 Precisely the same pattern of fluorescence was found following application of the three more specific conjugated antisera

DISCUSSION

That the present case was one of primary amyloidosis is in the first place made probable by the anamnestic data since our patient was completely well until the age of 65 years That year the fatal disease made its first appearance as a heart failure, quickly followed by symptoms of chronic renal disease and liver cirrhosis He never suffered from arthritis, nor did he ever have tuberculosis or other long standing infections

The autopsy findings too indicated amyloidosis in its "primary" form because of its distribution along the vessels, its appearance in the skin, etc The early cardiac involvement in primary amyloidosis has been stressed (*Symmers* 1956) The histochemistry too suggested primary amyloidosis, all the more or less specific amyloid reactions being only faintly positive except the Alcian blue reaction which showed moderate positivity As a common rule all of these reactions are considered unmistakably positive in most cases of "secondary" human amyloidosis (*Christensen* 1963)

The clinical course was not in support of a diagnosis of Waldenström's primary macroglobulinaemia There was no lymphadenopathy and no bleeding tendency Microscopic investigation of lymph nodes and bone marrow revealed nothing abnormal beyond the amyloid changes

By means of a direct Coon's technique *Dutcher & Fahey* (1960) were able to demonstrate "lymphocytoid plasmacells" as the sites of formation of the pathological IgM-paraprotein in a case of Waldenström's macroglobulinaemia Using the same technique and an anti-IgM antiserum we were unable to demonstrate specific fluorescence, neither in cells nor in the amyloid material

The significance of the renal tumour is open to dispute. It was of small size, showed no signs of invasive growth, let alone metastases Its malignancy was more than doubtful Cancer has been described as

causing paraproteinaemia (*Jirgensons & Cooper* 1956) and among 19 sera from patients with "secondary" paraproteinaemia *Waldenström* (1961) found 10 cases associated with cancer, two of these being paraproteinaemias of the IgM-type In most of these reports the cancer was in an advanced stage, often with skeletal metastases Though, one of these cases with pronounced secondary macroglobulinaemia is open to doubt, mainly because the alleged "primary" tumour was a very small bronchogenic carcinoma which was not recognized until at autopsy (*Laurell et al* 1957) Finally it should be remembered that the majority of patients with paraproteinaemias represent an age group in which tumours as casual findings at autopsy by no means are rare

Ask-Upmark (1940) reviewed 16 earlier reported cases of hypernephroma complicated by amyloidosis and postulated that the protracted course, the high sedimentation rate, and recurrent fever pointed to an "immunizing" effect of this kind of tumour, thus explaining its alleged higher rate of "secondary" amyloidosis This assumption has only partly been confirmed in later reports *Kimball* (1961) found 16 cases (0 4 per cent) of amyloidosis among 4,033 cases of cancer Only one case of amyloidosis was considered secondary to renal carcinoma *LeCoulant et al* (1961) found cancer in 2–4 per cent of patients with amyloidosis, most frequently hypernephroma *Dahlin* (1949) reported 5 cases of cancer among 30 cases of secondary amyloidosis But, as is the case in paraproteinaemias, simple coincidence cannot be excluded

In the present case we find it justified, from a patho-physiological point of view, to link the IgM paraproteinaemia to the amyloidosis and regard the renal tumour as an incidental finding

As already mentioned the four cases of primary amyloidosis reported by *Clausen & Christensen* (1964) seem to be the first in which a benign paraproteinaemia has been shown to accompany primary amyloidosis In earlier reports mainly dealing with primary amyloidosis the presence of an abnormal band between α_2 and β in the electrophoresis strip has been emphasized In some reports this band has even been considered of diagnostic value (*Rukavina et al* 1956, *Chambers et al* 1958, *Sallman et al* 1960). It is very probable that the described band in reality represented a paraprotein corresponding to those described by Clausen & Christensen It should be mentioned that Clausen & Christensen's four patients represented four consecutive cases of primary amyloidosis and that we since then, in this institute have seen two more patients with typical primary amyloidosis associated with an IgG paraproteinaemia We therefore find it very likely that thorough investigations will reveal some kind of paraproteinaemia in the majority of patients with systemic, primary amyloidosis

It is of course not possible to decide whether the paraproteinaemia is secondary to the amyloidosis or vice versa The third possibility *viz* that they both represent parallel manifestations of the same underlying disorder will de discussed later in this paper In the present state

of our knowledge it is impossible to offer any explanation of the first possibility As "essential", monosymptomatic paraproteinaemias in otherwise healthy people are known to be able to persist for years (*Waldenstrom* 1961) the second possibility cannot be ruled out

Varying degrees of bone marrow plasmocytosis have been described in connection with primary amyloidosis (*Teilum* 1948, *Conn & Sundberg* 1961, *Kyle & Bayrd* 1961) Neither in the present case, nor in the four cases reported by Clausen & Christensen, however, the number of bone marrow plasma cells were increased In the American as well as in the French literature the frequency of amyloidosis complicating multiple myeloma ("paramyloidosis") is reported to range between 10 and 25 per cent It is noteworthy that these figures never have been reproduced in this country *Drivsholm* (1965) found no cases of amyloidosis among 105 patients with multiple myeloma

Apitz (1940) postulated that an occult or overt malignant plasmocytoma was underlying each case of primary amyloidosis, but this author failed to produce convincing evidence in favour of this hypothesis *Osserman* (1959) and *Osserman et al* (1964) regard a so called "plasmocytic dyscrasia" as an initial stage in the pathogenesis of amyloid disease This "dyscrasia" might give rise to the formation of abnormal proteins of the Bence-Jones type which after leaving the blood stream were to precipitate in the tissues as insoluble complexes with local tissue proteins or mucopolysaccharides—in reality a modification of the Apitz theory This theory of "paraproteinosis" has been substantiated to some degree in the German literature (*Randerath* 1950, *Schneider* 1955, *Letterer* 1959) It operates with the assumption that the amyloid substance is formed from a circulating amyloid precursor oozing out through the capillary walls ("Paraproteinspeicherung") The immunohistochemical demonstration of γ-globulin in human amyloid material has been taken in support of the assumption of a local precipitation of antigen antibody nature (*Mellors & Ortega* 1956 *Vazquez & Dixon* 1956) The theory, however, does not explain the localization of the amyloid substance The presence in this substance of antigenic determinants identical with some of the γ-globulins does not support it either, mainly because of the varying composition of amyloid from case to case In our case we were unable to reproduce the findings obtained by Mellors & Ortega and Vazquez & Dixon

On the assumption that paraprotein were the amyloid precursor it seems unlikely that paraproteins with physicochemical and immunological properties which differ to such a degree as those of the three immunoglobulins IgG, IgA and IgM, should be able to play the same rôle in the pathogenesis of the primary amyloidosis

Based on studies of experimental and human secondary amyloidosis *Teilum* (1952, 1964) was able to offer a more natural explanation He regards the amyloid deposits as a local cellular phenomenon—a cytochemically well defined dysfunctional phase in the protein synthesizing

function of reticulo endothelial cells The break-down of this function in the face of persistent stimulation then results in a more or less general perversion of protein synthesis leading to precipitation of glycoprotein-like substances *in situ*.

Using this concept in the explanation of the pathogenesis of primary amyloidosis the same mechanism might be assumed to act in other "clones" of mesenchymal cells, *i e* those producing immunoglobulins, thus giving rise to the formation of more or less "normal" globulins—the paraproteins.

This would explain the amyloidosis and the accompanying paraproteinaemia as two parallel manifestations of the same underlying disturbance of mesenchymal tissue function

SUMMARY

The first case of an IgM-paraproteinaemia ("secondary" macroglobulinaemia) associated with a systemic, primary amyloidosis is reported The diagnosis was established after liver and rectum biopsies, and verified at autopsy The localization was typically "primary", mainly to the heart, the vessels and the skin Immunohistochemical investigations failed to confirm earlier reports on the presence of immunoglobulins in the amyloid substance

The paraprotein was identified by means of immunoelectrophoresis and Tiselius electrophoresis It was almost identical with that of Waldenström's disease, but the patient showed no other evidence of this disease The lymph nodes and the bone marrow were microscopically normal, except for the amyloid changes

The investigations are discussed and the literature reviewed The pathogenesis is discussed in light of Teilum's earlier works

REFERENCES

Apitz K Die Paraproteinosen (Über die Störung des Eiweissstoffwechsels beim Plasmocytom) Virchow Arch Path Anat *306* 631 1940

Askanazy pmark E On amyloidosis induced by tumours of the kidney Acta med scand *104* 512 1940

Braunsteiner H Oswald E Pakesch F & Reimer E Lymphoretikulosen mit Makroglobulinämie Wien Z inn Med 37 349 1956

Chambers R A Medd W E & Spencer H Primary amyloidosis with special reference to involvement of the nervous system Quart J Med 27 207 1958

Christensen H E Amyloidosestudier Thesis Copenhagen 1963

Clausen J & Christensen H E P-
mary amyloidosis Acta

Conn R B jr & Sundberg R D
39 61 1961

Coons A H & Kaplan M H Localization of antigen in tissue cells J exp Med 91 1 1950

Dahlin D C Amyloidosis Proc Staff Meet Mayo C

Dripsholm Aa Myelomatose

Dutcher T F & Fahey J L intranuclear localization of 18 S gamm of Walden
strom Proc Soc exp Bio

Fessel W J Clinical analysis of 142 cases with high molecular weight serum proteins Acta med scand., suppl 391 1962
Gothoni G Wasastjerna C & Jeglinsky B Macroglobulinaemia primary (Waldenstrom) and symptomatic in rheumatoid arthritis Acta med scand *177* 263 1965
Jannes J Saris N E Kosonen T Makela, T & Seppala P Makroglobulinemi Nord Med *65* 695 1961
Jirgensons B & Cooper J A Abnormal globulins in some neoplastic diseases Clin chim Acta *1* 16 1956
Kimball K G Amyloidosis in association with neoplastic disease Ann int Med *55* 958 1961
Kyle R F & Bayrd E D Primary systemic amyloidosis and myeloma Arch int Med *107* 344 1961
Laurell C B Laurell H & Waldenstrom J Glycoproteins in serum from patients with myeloma macroglobulinemia and related conditions Am J Med *22* 24 1957
LeCoulant P Texier L Maleville J & Fauret Y Maladie amyloide et cancer Path Biol *9* 1587 1961
Letterer E In Allgemeine Pathologie Grundlagen und Probleme Fin Lehrbuch p 237 G Thieme Stuttgart 1959
Mandema E De makroglobulinaemie van Waldenstrum Ned Tschr Geneesheilk *98* 2109 1954
Mellors R C & Ortega L G Analytical pathology III New observations on the pathogenesis of glomerulonephritis lipid nephrosis periarteritis nodosa and secondary amyloidosis in man Am J Path *32* 455 1956
Nick J Contamin F Brion S Guillard A & Mme Guiraudon Macroglobulinemie de Waldenstorm avec neuropathie amyloide Observation anatomo-clinique Rev Neurol (Paris) *109* 21 1963
Onal A & Cooper T Relation of bone marrow findings to serum protein changes in lymphosarcoma chronic lymphocytic leukemia and Hodgkins disease Blood *15* 114 1960
Osserman E F Plasma cell myeloma II Clinical aspects N Engl J Med *261* 1006 1959
Osserman E F Takatsuki K & Talal N The pathogenesis of amyloidosis" In Seminars in Hematology Fd Peter A Miescher Grune & Stratton Inc 1964
Randerath E Uber die Morphologie der Paraproteinosen Verh deutsch Ges Path *32 Tag* 27 1950
Rukavina J G Block W D Jackson C E Falls H F Carey J H & Curtis A -C Primary systemic amyloidosis a review and an experimental genetic and clinical study of 29 cases with particular emphasis on the familial form Medicine *35* 239 1956
Sallman L Kaufman H E Haase G R Bartter F C & Thomas L B Primary amyloidosis clinical staff conference at the National Institutes of Health Ann int Med *52* 668 1960
Scheiffarth F & Got H Die Bedeutung der Immunoelektrophorese bei der Differenzierung pathologischen Seren Int Arch Allergy appl Immunol *16* 61 1960
Schneider G Studien zur Korrelation der Eiweisskorper des Blutplasmas und der Organe bei experimenteller Dysproteinamie II Quantitative Anderungen des Eiweiss- und Stickstoffgehaltes von Leber Milz und Nieren im Verlauf einer experimentell erzeugen Dysproteinamie Virchows Arch path Anat *327* 354 1955
Seligman M Mais L & Bernard J Immuno electrophoretic analysis of sera from leukemic patients Abstract in Blood *15* 938 1960
Symmers W S Primary amyloidosis a review J clin Path *9* 187 1956
Teilum G Hyperglobulinemia periarterial fibrosis of the spleen and the wire loop lesion in disseminated lupus erythematosus in relation to allergic pathogenesis Am J Path *24* 409 1948
Teilum G Cortisone ascorbic acid interaction and the pathogenesis of amyloidosis Mechanisms of action of cortisone on mesenchymal tissue Ann rheum Dis *11* 119 1952
Teilum G Pathogenesis of amyloidosis Acta path et microbiol scandinav *61* 21 1964

Vazquez J J & Dixon F J Immunohistochemical analysis of amyloid by the fluorescence technique J exp Med *104* 727 1956

Waldenstrom J Clinical diagnosis and biochemical findings in material of 296 sera with M type narrow γm globulins Acta med scand *170* suppl 367 p 110 1961

Willi H Koller F & Raaflaub J Symptomatische Makroglobulinamie bei Lues congenita Acta haemat (Basel) *11* 316 1954

Acta path. et microbiol. scandinav. 66, 169–185, 1966

The Rheumatic Research Laboratory, University Institute of Pathological Anatomy,
(Head: G. Teilum) Copenhagen, Denmark

SARCOIDOSIS

1. *Ultrastructural Investigations on Epithelioid Cell Granulomas*

By

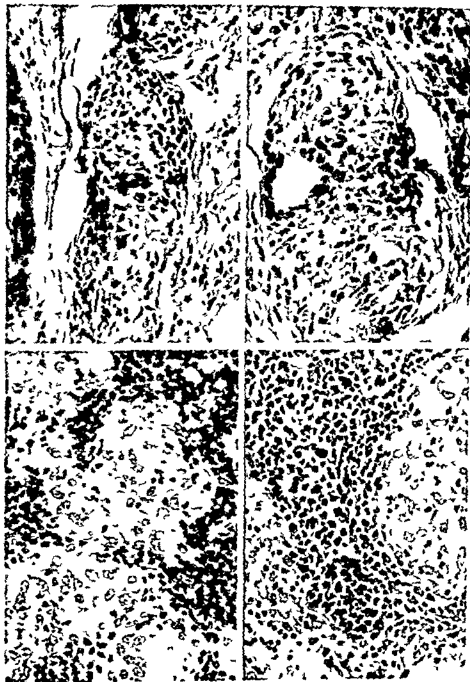
J. WANSTRUP and H. E. CHRISTENSEN

Received 31.vii.65

Sarcoidosis is defined as 'a disease of unknown aetiology and pathogenesis, characterized histologically by *generalized* epithelioid cell granulomas with inconspicuous or no necrosis and by the frequent presence of giant cells with or without vesicular or lamellated often calcified inclusion bodies'. Although certain clinical, radiological and serological findings may serve to establish the diagnosis, the demonstration of the epithelioid cell granuloma is considered a diagnostic imperative. That this characteristic lesion is built up of reticular cells of phagocyte type, which have taken up some foreign material of lipid nature and become "epithelioid", has long been a general assumption. However, several observations indicate that immunological or allergic cell mechanisms are included, but at present their relation to pathogenesis or aetiology is unknown. Further, the granuloma formation seems to pass through certain developmental stages terminating in hyalinosis and/or fibrosis.

In the past the fully developed granulomas have been in the centre of interest, while less attention has been paid to the earliest phases in the granulomatous lesion (*Barrie & Bogoch* 1953 and *Bellot* 1962). Recently morphogenetic investigations of early experimental granulomas were reported by us (*Wanstrup & Christensen* 1965) in order to study the pathogenesis of granuloma formation.

Ultrastructural studies on experimental granulomas of epithelioid type have been published (*Gusek* 1958–63 and *Bönicke, Fasske & Themann* 1963), but such investigations of human sarcoidosis are at present not available. The present study reports on ultrastructural findings in the granulomas in two cases of sarcoidosis.[1]



MATERIAL AND METHODS

The investigations are based on two verified cases of sarcoidosis (A and B)

Case A (RH H) was a 43 years old woman admitted to the dermatological department for widespread circinate elements in the cutis A biopsy showed a picture characteristic of sarcoidosis (Figs 1 2) The generalization of the lesion was confirmed by liver biopsy and serum electrophoresis revealed marked hyper gamma globulinaemia

Case B (RH TA) was a 59 years old woman with paresis of the facial nerve and eye symptoms lasting for 1½ year X ray revealed characteristic pulmonal infiltrations and biopsies from the cutis striated musculature liver and lymph nodes (Figs 3-4) all showed typical epithelioid cell granulomas

Biopsy material from the cutaneous lesions in case A and a para tracheal lymph node from B was taken for electron microscopic investigations The tissue was quickly cut in small pieces measuring about 1 cmm and fixed for two hours in buffered osmium tetraoxide (pH 7 2) dehydrated in acetone and embedded in Vestopal W For orientation and comparative study semifine sections about 1 µ thick were cut and stained with toluidine blue Finally ultra thin sections were cut on a LKB ultramicrotome They were after stained with uranyl acetate (5 per cent aqueous solution) and/or lead hydroxide according to the method of Reynolds for 1 2 hours and examined in a Siemens Elmiskop I Corresponding paraffin embedded tissue was cut in sections 6 µ thick and stained with haematoxylin eosin v Gieson methyl green pyronine and periodic acid Schiff (PAS) staining techniques

RESULTS

Light Microscopy

The histological picture was that of sarcoidosis in active stage *Case A* showed well demarcated cell rich clusters of reticular cells of epi thelioid type The granulomas were of different appearance The most cellular in Fig 1 contain many pyroninophilic (basophilic) cells, while the more advanced in Fig 2 are built up of rather few, larger cells some with varying degrees of cytoplasmic PAS positivity The lymph node from *case B* contains many fully developed epithelioid cell granulomas obviously formed by quite uniform, closely packed big cells with in distinct boundaries giving a syncytial appearance Sometimes mono nuclear cells were clearly demonstrated especially in the periphery of the granulomas but also interstitially between these Many cytoplasmic vesicular structures are seen and also veritable asteroid bodies (Fig 3) A lively proliferation of different reticular cells is found in the cell

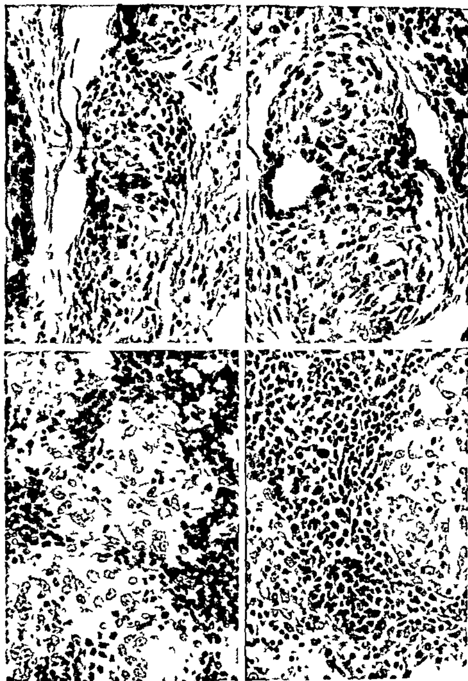
---

Fig 1 Young cell rich epithelioid cell granuloma from cutaneous biopsy in case A Several cells of the plasmocytic range with varying degrees of pyronino philia Paraffin section H E stain × 285

Fig 2 Larger mature granulomas from the same biopsy as Fig 1 Large reticular cells in granulomatous and syncytial arrangement many with cytoplasmic PAS positivity Typical Langhans giant cell Paraffin section PAS stain × 285

Fig 3 Typical epithelioid cell granulomas from lymph node in case B The cyto plasm is abundant with many vesicular formations A veritable asteroid body in giant cell near the centre Paraffin section H E stain × 285

Fig 4 Intergranulomatous field from lymph node Lively proliferating reticular cells in various stages of differentiation Many large cells with PAS positive cytoplasm are seen In the lower part a granuloma like collection of pyro ninophilic cells Paraffin section PAS stain × 285



rich interstitial tissue (Fig 4) Some of these cells are strongly pyroninophilic and collected in granuloma like clusters while others are larger reticular cells with cytoplasmic PAS positivity

The semifine sections from Vestopal embedded tissue show a more varied and detailed picture Fig 5 corresponds to the ordinary prepared section in Fig 4 with the cell rich inter granulomatous field showing many plasmatoid cell types of different differentiation Transitional stages from the primitive reticulum cell via the activated reticulum cell of *Marshall & White* and the basophilic reticulum cell to the preplasmocyte and the mature plasmocyte are represented Also larger immature cells identical to the weakly PAS positive cells in Fig 4 are seen From the latter there seems to be a gradual transition to epithelioid cells characterized by relatively scattered nuclear chromatin a dense central nucleolus and abundant cytoplasm often with indistinct boundaries These cells are similar to those within the granulomas especially if compared with the young cell rich granulomas from case A (Fig 6) Fig 7–8 show epithelioid cell granulomas from the lymph node in case B The epithelioid cells have pale, vesicular, uniform nuclei often with a centrally placed nucleolus Cell boundaries are indistinct and the cells are closely packed this combination gives a syncytial impression Real giant cells of both Langhans and Touton type are also found The cytoplasm is abundant and stains with varying intensity In many cells a perinuclear darker area is present corresponding to a PAS positive reaction in paraffin sections Many cytoplasmic vesicular structures are found especially in the giant cells but also in the mononuclear epithelioid cells (Fig 7) To the right in Fig 7 epithelioid cells with one, two and three nuclei are seen Fig 9 shows the margin of a granuloma with degenerating epithelioid cells with loss of contours resulting in the formation of a hyaline like area A network of capillaries with swollen endo and perithelial cells is seen just outside the granulomas (Fig 7–8)

Fig 5 Cell rich intergranulomatous field from lymph node (compare Fig 4) Different types of reticular cells Many cells of the plasmocytic range and greater immature epithelioid cells corresponding to those within the granulomas Semifine section Toluidine blue stain × 560

Fig 6 Loosely arranged cell rich granuloma from the cutis in case A Epithelioid cells with large chromatin weak slightly indented vesicular nuclei Abundant granulated cytoplasm with varying staining intensity and small cytoplasmic vesicles Cell boundaries indistinct Semifine section Toluidine blue stain × 560

Figs 7 8 Granuloma from lymph node with Langhans and Touton giant cells Note the varying staining intensity of the cytoplasm often with central para nuclear darker areas To the right in Fig 7 epithelioid cells with one two and three nuclei are seen Semifine section Toluidine blue stain × 380

Fig 9 Margin of granuloma from lymph node Degenerating epithelioid cells with loss of contours and fragmentation of nuclei resulting in the formation of a hyaline like area Semifine section Toluidine blue stain × 560



Fig 10

Activated primitive reticular cell with slightly lobulated nucleus distinct large nucleolus and mainly marginally situated chromatin Few well structured mitochondria and slightly dilated ergastoplasma (er) Ribosomes mostly as free granules diffuse or in small rosettes × 20000

Electron Microscopy

The ultrastructural findings in the younger cutaneous granulomas and in the more advanced ones from the lymph node revealed no principal differences and will be described collectively

Cells of the plasmocytic range mostly the more immature forms are frequently found within the granuloma and in close relation to it The same is true for primitive reticular cells with large lobulated



Fig 11

Closely packed reticular cells from margin of granuloma in lymph node To the left a primitive activated reticulum cell To the right a mature plasma cell with characteristic lamellated ergastoplasm, many fixed granules and prominent Golgi zone (G) × 20000

nuclei, relatively sparse marginally situated nuclear chromatin, and one or two prominent nucleoli, especially within the young cell-rich granulomas and peripherally in the more advanced ones The cytoplasm is moderate in amount with only few well-structured mitochondria Ribosomes are mostly seen as free granules diffusely or in small rosette-like formations Some of the often slightly dilated ergastoplasmic vesicles, however, are coated with fixed granules The Golgi zone is rarely seen, and if present, it is small Sometimes the perinuclear space shows dilated segments The cell surface is irregular with many clumsy protrusions No signs of phagocytic activity are seen in these cells Such activated cell types are illustrated in Figs 10–13 To the right in Fig 11 a mature plasma cell is seen closely packed against a primitive, activated reticulum cell The plasma cell contains a well developed Golgi zone and membranes of ergastoplasm possessing numerous ribosomes Fig 12 also shows a mature plasma cell, but here the ergastoplasm is moderately dilated and the number of ribosomes relatively decreased due to accumulation of secretion material in the form of a finely granular substance The nucleus is round and eccentric with characteristic arrangement of the rather



Fig 12

Mature plasma cell with abundant dilated ergastoplasm filled with a finely granular material Well structured mitochondria and characteristically arranged nuclear chromatin × 17500

abundant chromatine substance Fig 13 illustrates an activated more advanced reticular cell of epithelioid appearance The cytoplasm is relatively more abundant and the nucleus is large and vesicular with sparse chromatin arranged peripherally under the nuclear membrane The perinuclear space *still shows small dilatations The cytoplasmic* organelles have increased markedly in number and size Many large vesicular mitochondria are seen with few, irregularly arranged cristae *and sometimes the limit is blurred or incomplete Most of the ergas* toplasm is markedly dilated and contains loose, finely granular material There are many free and fixed granules and a prominent *Golgi zone In paranuclear position a peculiar lamellated structure* is seen It is composed of many uniform, curved bodies sometimes with a little "vesicular" swelling and resembling two closely packed thin *membranes* They have no fixed granules, but in the interstice between them are free ribosomes in small rosettes The nature and origin of this structure is unknown, —it bears no resemblance to a transversely cut Golgi zone

Figs 14–15 show fully developed epithelioid cells from the centre of the granulomas The nuclei have the same appearance as those of activated and basophilic reticulum cells, *i e* rather large vesicular and slightly lobulated nucleus with sparse, marginally placed chro-



Fig 13

Young reticular cell of epithelioid appearance from a cutaneous granuloma Large mitochondria (M) with few cristae and dilated vesicular ergastoplasm (er) with loosely granular content and many fixed granules Prominent Golgi zone (G) and centrally in the picture a peculiar lamellated structure (LS) × 28000

matin substance and one or two prominent nucleoli Sometimes less electron dense nuclear inclusion bodies are seen (Fig 14) The cytoplasm is abundant with a tremendous mass of organelles Many diffusely spread mitochondria are present Especially the largest have rather few, disorderly arranged cristae and contain often strongly osmiophilic small round bodies from one to three in number (Figs 14–15) The ergastoplasm is rather sparse and vesicular with varying



Fig. 13

Epithelioid cell from granuloma in lymph node. Prominent nucleolus and small intra nuclear bodies (arrows). Several mitochondria with rather few disorderly aranged cristae and often with strongly electron dense small bodies from one to three in number (mb). An abundance of well demarcated vesicular filled with a finely uniform granular material. Sparse vesicular ergastoplasm (er) with few fixed granules. In lower left corner a fibrillar collagen substance (cl) in close relation to the cell surface. × 20000

degree of dilatation. The amount of free and fixed granules is moderate or sparse. Golgi zones are prominent and small-vesicular. No accumulation of secretory products is seen within this organelle. Furthermore, an abundance of special vesicular structures is present. They are round and smooth and the majority has the size of mitochondria or smaller.



Fig 15

Epithelioid cell from lymph node Many mitochondria and vesicular ergastoplasm with rather few ribosomes Small electron dense mitochondria-like bodies Larger most often well-demarcated vesicles V filled with a granular material In some the surrounding membrane is blurred or absent (arrows and the content is of varying density × 20000

and most often they are well demarcated by a single membrane Some are larger and often the membrane is incomplete or absent The content is finely granular in loosely uniform arrangement and with varying but preferably weak or moderate osmiophilia They have no ribosomes attached to the outside and are diffusely spread in the cytoplasm apparently without special arrangements or constant relation to other



Fig 16

Section of giant cell from lymph node The nuclear and cytoplasmic pattern is just similar to that of the epithelioid cells Note the big area with Golgi zone structures (G) in the centre of picture × 20000

organelles However, a minority of such structures shows remnants of a double contoured membrane and contains, besides the granular material, strongly electron dense bodies or lamellated figures and crista-like shadows Consequently they bear some resemblance to altered mitochondria (Fig 18) They are most numerous in the advanced epithelioid cells and the giant cells Furthermore these cell types contain many highly characteristic lamellated structures known as myelin figures and in relation to these another strongly osmiophilic complex



Fig 17

Section of giant cell from lymph node Large area with concentric lamel

Insert × 30000

structure with pronounced vacuolization and sequestration (Fig
The giant cells have the same character as the mononuclear epithel
cells described above Fig 16 shows a section of a giant cell In
centre a huge area of small-vesicular and lamellated Golgi zone st
tures is seen This area *may* represent fusion of Golgi zones f

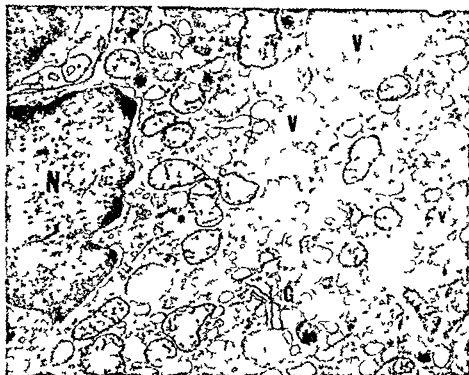


Fig 18

Epithelioid cell from cutaneous granuloma. Characteristic vesicular chromatinweak nucleus (N). An abundance of cytoplasmic structures. Many vesicular bodies (V) of varying size and with granular content. Several with dense sometimes partly double contoured surrounding membranes and cristae like shadows (arrows). Very few ribosomes and small vesicular Golgi zone (G). × 15000

several epithelioid cells which by coalescence have formed the multinucleated giant cell. The cells in the granuloma were generally closely packed and often the cell surfaces showed elongations in the form of slender villi-like protrusions interdigitating with those of adjacent cells. Sometimes a fibrillar collagen substance was seen in close relation to the epithelioid cells (Fig 19).

COMMENTS

The present study is a preliminary report containing the outlines of the cytology and the ultrastructure of the epithelioid cell granuloma in sarcoidosis. Some of the findings in a number of investigations of different experimental granulomas of epithelioid cell type (*Gusek* 1962) have been confirmed in human material. The epithelioid cells seemed morphologically to be highly active cells with an abundance of cytoplasmic organelles and other signs of lively metabolic activity. They are in general well structured and thus it is unlikely that the epithelioid cell in itself represents an inactive or burned out cell. Ultrastructurally the mononuclear epithelioid cells and the giant cells

show identical structure, which may indicate that the latter are formed by fusion of the former The large are i with Golgi zone structures in Fig 16 and observations by *Gusek* 1962 might favour such a morphogenetic mechanism So far the findings in sarcoid granulomas correspond to those of *Gusek* and others in different kinds of *experimental* granulomas (tuberculosis, lepra, beryllium or different "allergic granulomas") However, they stressed that epithelioid cells were highly active cells of phagocyte type, because many of them contained phagocytized corpuscular elements (leucocytes, erythrocytes, bacteria) or other solid substances situated in small vesicular structures Such a phagocytotic activity was also predictable both in the tuberculous granulomas with their caseous necrosis (and bacteria) and also in the other experiments, because the tissue to be investigated was taken locally at the site of injection of foreign material Other German workers (*Bönicke, Fasske & Themann* 1963) partly confirmed such findings in a model for "generalized" granulomas produced by intravenous injections of a heat killed tuberculous culture suspended in paraffin oil (Freund type adjuvant) This resulted in multiple paraffin oil emboli in the small pulmonal capillaries and subsequent development of a granulomatous lesion around the antigen-containing droplet They also found the epithelioid cell of "phagocyte type", but described a further type of plasmacellular appearance This is an important observation because it combines granuloma formation with reticular cells of the plasmocytic range and morphological signs of antibody formation

The present investigations of sarcoid granulomas revealed many plasmatoid cell types, especially the more immature forms Several reticular cells of the activated or basophilic type as seen under immunization (*Fagraeus* 1948, 1958, *Stoeckenius & Naumann* 1957 and *Stoeckenius* 1958) were present within the granulomas or in close relation to these Epithelioid cells from the cell-rich cutaneous granulomas (Fig 13) retain the appearance of immature reticular cells, and the same is partly true with regard to the more advanced epithelioid cells No definite signs of phagocytic activity were seen in the present material (cfr see below) The dense complex bodies with pronounced vacuolization (Fig 17) might represent an advanced stage of erythrophagocytosis (*Essner* 1960) but earlier phases of this process were not observed and staining for iron on semifine sections remained negative Both these structures and the myelin figures are well known and frequent, non specific findings in both epithelial and mesenchymal cells under a varity of noxious or even physiological stimuli (*Hruban et al* 1963), but their deeper nature and mode of origin are at present unknown, although it is generally accepted that myelin figures in one way or another are connected with lipid metabolism According to *Hruban et al* abnormalities in either protein or cholesterol synthesis appear to contribute to myelin membrane formation

A special cytoplasmic structure of interest is the often well-demarcated and abundant vesicular bodies filled with a loosely granular material Such structures have been frequently described in the past under different names (microsomes, cytosomes, lysosomes) (*Rhodin* 1954, *Gansler & Rouiller* 1956, *Rouiller & Bernhard* 1956 and *Bennett* 1956) but their real nature and origin is unknown The question in focus is whether they represent phagocytized material or secreted substances *Gusek* considers them under the name of cytosomes—in connection with other findings—as a sign of phagocytosis They bear some resemblance to Russell body-like structures as described among others by *Dohi, Hanaoka & Amano* 1957 and *Welsh* 1960 A minority of these bodies, however, contains remnants of mitochondrie-like structures (Fig 18) and thus possibly represent sequestered or degenerated mitochondria This again rises the *old* problem about splitting of mitochondria and granulation of different mesenchymal cells (plasma cells, mast cells) The question raised in the present paper about the meaning of the special cytoplasmic structures cannot be answered with certainty, but is seems as if reticular cells of the appearance of antibody producing cells are connected in one way or another with the granuloma formation in sarcoidosis The possible rôle of phagocytosis —in spite of the lack of definite signs of such phenomena—needs further investigations, and studies dealing with the unexplained cytoplasmic structures and also hyalinosis-fibrosis are in progress

SUMMARY

The present paper is a presentation of the ultrastructural findings in epithelioid cell granulomas in the cutis and in a paratracheal lymph node from two cases of verified generalized sarcoidosis Special emphasis is laid upon the cell types present within the granulomas and in their immediate neighbourhood The epithelioid cells proper had to some extent the appearance of activated and basophilic reticular cells In addition to this they contained many cytoplasmic bodies composed of a finely granular, moderate osmiophilic material It is discussed whether this material could be a secretion product or whether it represents phagocytized substances The authors favour the opinion, that the former possibility best explains the morphologic picture of the ultrastructure It is concluded, that these cells are, or have been highly active synthesizing cells The giant cells showed the same ultrastructure as the epithelioid cells and are presumably formed by fusion of these Mitochondrial degeneration and other forms of focal cytoplasmic changes (myelin figures) were often found Within the granulomas but especially around them all transitional developmental stages from the primitive reticular cell to the mature plasma cell were seen, and the authors believe, that these findings represent an important pathogenetic relationship to the special granulomatous lesion Fibrosis

and hyalinization was observed, but this together with further studies of the unexplained cytoplasmic structures will be reported in subsequent papers

REFERENCES

1 *Barrie H J & Bogoch A* The natural history of the sarcoid granuloma Am J Path 29 451 469 1953

2 *Bell ,t S M* De jonge fasen van de granulomen bij de ziekte van Besnier Boeck Schaumann Amsterdam 1962

3 *Bennett H S* A suggestion as to the nature of lysosome granules J Biophysic and Biochem Cytol 2 suppl 185 1956

4 *Bonicke R Fasske E & Themann H* Submikroscopische und enzymhisto chemische Beitrage zur formalen Genese des Epitheliodzellgranuloms Klin Wchnschr 41 753 768 1963

5 Committee on Sarcoidosis National Academy of Sciences National Research Council 1960

6 *Dohi S Hanaoka M & Amano S* Electron microscopic studies on the plasma cell Acta Path Japon 7 1 11 1957

7 *Essner E* An electron microscopic study of erythrophagocytosis J Biophysic and Biochem Cytol 7 329–334 1960

8 *Fagraeus A* Antibody production in relation to the development of plasma cells Acta Med Scand Suppl 204 1948

9 *Fagraeus A* Cellular react on in antibody formation Acta Haemat (Basel) 20 1 8 1958

10 *Gansler H & Rouiller C* Modifications physiologiques et pathologiques du chondriome Schweiz Z Path Bakt 19 217 243 1956

11 *Gusek W* Die Feinstruktur der einkernigen Makrophagen und der mehrkerni gen Riesenzellen im Fremdkorpergranulationsgewebe Frankf Z Path 69 429 436 1958

12 *Gusek W* Uber die Ultrastruktur und Natur der Epitheloidzellen Frankf Z Path 69 685–694 1959

13 *Gusek W* Submikroscopische Untersuchungen zur Feinstruktur aktiver Binde gewebezellen Gustav Fischers Verlag Stuttgart 1962

14

15

16 National Research Council (Division of medical sciences) *Bulletin Sarcoidosis* 1 11 1948

17 Proceedings of the international conference on Sarcoidosis June 1 3 1960 Am Rev Resp Dis 84 (Part 2) 1 183 1961

18 Proceedings of the Third International Conference on Sarcoidosis Stockholm Sept 11 14 1963 Acta Med Scand Suppl 425 1964

19 *Rhodin J* Correlation of ultrastructural organisation and function in normal and experimentally changed proximal convoluted tubule cells of the mouse kidney An electron microscopic study including an experimental analysis of the conditions for fixation of the renal tissue for high resolution electron microscopy Stockholm 1954 Aktiebolaget Godvil

20 *Rouiller C & Bernhard W* "Microbodies and the problem of mitochondrial regeneration in liver cells J Biophysic and Biochem Cytol 2 355–360 1956

21 *Stoeckenius W & Naumann P* Elektronmikroskopische Untersuchungen zur Antikorperbildung in der Milz Transact 6th Congres Europ Soc Haemat 1957

22 *Stoeckenius W* Weitere Untersuchungen am lymphatischen Gewebe Verh deutsch Gesellsch Path 42 Tag 304 311 1958

23 *Wanstrup J & Christensen H E* Granulomatous lesions in mice produced by Freunds adjuvant Morphogenesis and phasic development Acta path et microbiol scandinav 63 340–354 1965

24 *Welsh R A* Electron microscopic localization of Russell bodies in human plasma cell Blood 16 1307 12 1960

A special cytoplasmic structure of interest is the often well-demarcated and abundant vesicular bodies filled with a loosely granular material Such structures have been frequently described in the past under different names (microsomes, cytosomes, lysosomes) (*Rhodin* 1954, *Gansler & Rouiller* 1956, *Rouiller & Bernhard* 1956 and *Bennett* 1956) but their real nature and origin is unknown The question in focus is whether they represent phagocytized material or secreted substances *Gusek* considers them under the name of cytosomes—in connection with other findings—as a sign of phagocytosis They bear some resemblance to Russell body like structures as described among others by *Dohi, Hanaoka & Amano* 1957 and *Welsh* 1960 A minority of these bodies, however, contains remnants of mitochondria-like structures (Fig 18) and thus possibly represent sequestered or degenerated mitochondria This again rises the *old* problem about splitting of mito chondria and granulation of different mesenchymal cells (plasma cells, mast cells) The question raised in the present paper about the meaning of the special cytoplasmic structures cannot be answered with certainty, but is seems as if reticular cells of the appearance of antibody producing cells are connected in one way or another with the granuloma formation in sarcoidosis The possible rôle of phagocytosis —in spite of the lack of definite signs of such phenomena—needs further investigations, and studies dealing with the unexplained cytoplasmic structures and also hyalinosis-fibrosis are in progress

SUMMARY

The present paper is a presentation of the ultrastructural findings in epithelioid cell granulomas in the cutis and in a paratracheal lymph node from two cases of verified generalized sarcoidosis Special emphasis is laid upon the cell types present within the granulomas and in their immediate neighbourhood The epithelioid cells proper had to some extent the appearance of activated and basophilic reticular cells In addition to this they contained many cytoplasmic bodies composed of a finely granular, moderate osmiophilic material It is dis cussed whether this material could be a secretion product or whether it represents phagocytized substances The authors favour the opinion, that the former possibility best explains the morphologic picture of the ultrastructure It is concluded, that these cells are, or have been, highly active synthesizing cells The giant cells showed the same ultrastructure as the epithelioid cells and are presumably formed by fusion of these Mitochondrial degeneration and other forms of focal cytoplasmic changes (myelin figures) were often found Within the granulomas but especially around them all transitional developmental stages from the primitive reticular cell to the mature plasma cell were seen, and the authors believe, that these findings represent an important pathogenetic relationship to the special granulomatous lesion Fibrosis

An increased concentration of the abovementioned enzymes (Table 1) has been demonstrated in the early phase of wound healing in skin of 14 rats (7 males and 7 females) and 14 guinea pigs (7 males and 7 females)

RESULTS

All the O C T no 1 embedded sections appeared to become better than the routine sections as the wound edges were found better preserved as well as it was possible to obtain thinner sections presenting no folds There was no difference in the staining between the two groups of wounds especially no reduction in the stainability among the O C T no 1 treated tissues Untreated sections appeared thicker with a variable number of folds and irregularities which complicated the evaluation of the amount of enzyme present and of the wound healing processes

CONCLUSION

O C T no 1 embedding material is considered an improvement in frozen tissue techniques as sections are not folded and can be cut thinner The new embedding material is not interfering with the demonstration and with the visible amount of alkaline phosphatase acid phosphatase, aminopeptidase, unspecific esterase and adenosintriphosphatase examined by the histochemical methods referred

REFERENCES

1 *Davis B J & Ornstein L* High resolution enzyme localization with a new diazo reagent hexazonium pararosaniline J Histochem Cytochem 7 297 1959

2 *Grogg F & Pearse A G E* Coupling azo dye methods for histochemical demonstration of alkaline phosphatase Nature *170* 578 1952

3 *Grogg F & Pearse A G E* A critical study of histochemical techniques for acid phosphatase J Path Bact *64* 627 1952

4 *Nachlas M M Monis B Rosenblatt D & Seligman A M* Improvement in the histochemical localization of leucine aminopeptidase with a new substrate L leucyl 4 methoxy 2 naphthylamide J Biophys Biochem Cytol 7 261 1960

5 *Padykula H & Herman E* The specificity of the histochemical method for adenosin triphosphatase J Histochem Cytochem *3* 161 1955

Acta path et microbiol scandinav 66 188 196 1966

The University of Bergen School of Medicine, The Broegelmann Research Laboratory for Microbiology, Bergen, Norway

HETEROGENEITY OF ANTI-ANTIBODIES IN INDIVIDUAL HUMAN SERA

By

JACOB B NATVIG

Received 20.viii.65

In a previous paper heterogeneity of anti-antibodies (Milgrom-Grubb type) in different sera was demonstrated (18) Three patterns of reactions of the anti-antibodies were described The first type of sera only agglutinated red cells sensitized by Gm(f) anti-D Sera showing the second type of reaction agglutinated red cells sensitized by Gm(a) or Gm(f) anti-D Serum of the third type agglutinated red cells sensitized by any of the anti-D sera specific for Gm(a), Gm(b) or Gm(f)

In addition, certain findings indicated multiple specificities of anti-antibodies in individual sera This was also recently suggested by *Harboe et al* (10, footnote p 508) These observations led to further studies on the heterogeneity of anti-antibodies

MATERIALS AND METHODS

Red cells O R_1R_2 (CDe cDE) red cells from a single donor were stored at 4° C in acid-citrate dextrose solution for a maximum of 5 days (16)

Anti antibody sera Eight of the sera Gm typed and used in a previous study (18) were selected The sera contained anti γG globulin factors showing definite anti antibody reactions, which were not inhibited either by pooled native γ globulin or by native γ globulin in sera from which γG antibodies in immune (antigen anti body) complexes reacted readily

Incomplete anti D sera The anti D sera 3066 3175 and 3311 had been used previously in a panel of sera (18) The reference anti D sera (3091 3101 and 3083) were also included Anti D 3091 and 3311 from Gm(a+x+b−f−) and Gm(a+x b+f+) donors were specific for Gm(a) and Gm(x) and Gm(a) respectively Sera 3101 3083 and 3066 were from Gm(a−x b+f+) donors Anti D 3101 was specific for Gm(b) but contained a minor proportion of Gm(f) molecules detectable only by absorption (16) Anti D 3083 and 3066 were specific for Gm(f) but anti D 3066 contained a minor proportion of Gm(b) molecules (16) Anti D 3175 from a Gm(a+x b f+) donor contained Gm(a) and Gm(f) molecules in a proportion of about 1 1 but no Gm(b) anti D molecules were demonstrated (17) Anti CD Ripley was used because of its polyspecificity in detecting anti γ globulin factors (3 22)

Anti Gm sera and normal sera All sera had been used in earlier investigations (17 18

Anti human gammaglobulin serum Rabbit antiserum (K 980) against whole human serum was used as previously (16)

Aided in part by a grant from *L. Meltzers høyskolefond* Bergen Norway

Gammaglobulin Human gammaglobulin 1? per cent solution batch 807/9 was kindly provided by AB Kabi Stockholm Sweden A one per cent sample of this

Agglutination tests (16) Agglutinating activity of anti antibodies was tested with red cells sensitized by excess amounts of incomplete anti D according to the tube and slide techniques described previously The sensitized red cells were tested by

ime of the
absorption

ra specific
were pre
tibody and
sensitized
a smaller
ature for 2

hours From one to 3 subsequent absorptions were performed

EXPERIMENTS AND RESULTS

The 3 types of agglutination pattern of the anti antibodies are shown in Table 1

TABLE 1
Agglutination Patterns of the Different Types of Anti Antibodies

Anti antibody	Serum	Red cells sensitized by anti D specific for		
		Gm(a) (3091)	Gm(b) (3109)	Gm(f) (3083)
Type I	(i e 55 647)	—	—	+++
Type II	(i e 356 649)	+++	—	+++
Type III	(141)	+++	++	+++

Absorption of Anti Antibodies of Type I

Red cells sensitized by Gm(f) anti D 3083 or by anti D 3175 removed anti antibody 55 (from a Gm(a—x—b+f+) donor) in one absorption (Fig 1) Red cells sensitized by Gm(b) anti D 3109, containing a few *Gm(f) molecules, were not agglutinated by anti antibody 55 but re*moved it in 3 absorptions The activity of serum 55 was not removed by using Gm(a) anti D 3091, and the remaining activity was not inhibited by one per cent pooled native γ globulin or by native γ globulin from the anti D serum used for the detection of the anti antibody (cfr 18 Tables 3 and 4)

Serum exhausted of activity by using Gm(f) anti D was tested against red cells sensitized by each of two Gm(f) anti D sera (3066 and 3083) by anti D 3175 or by anti CD Ripley No reaction was

Symbols and activity in agglutination reactions

		Anti Gm(a)	Anti Gm(b)	Anti Gm(f)	Anti-antibody 55
■	Anti-D 3083	—	—	+++	+++
□	Anti-D 3109	—	+++	—	—
▲	Anti-D 3091	+++	—	—	—
△	Anti-D 3175	+++	—	—	+++

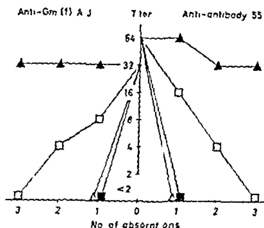


Fig. 1

Absorption of anti-antibody 55 (Type I) and anti Gm(f) A.J with red cells sensitized by different anti D sera. Each serum diluted 1 : 2 was absorbed 3 times by an equal volume of packed sensitized red cells. After each absorption the activity of the supernates was tested against red cells sensitized by Gm(f) anti D 3083. The activities of the sensitized red cells were tested in agglutination reaction before use in absorption. — no agglutination, +++ strong agglutination

obtained with any of these sensitized red cells. Absorptions by using another Gm(f) anti-D serum gave similar results as when Gm(f) anti-D 3083 was used.

Two other anti antibody sera of Type I (576 and 647, from Gm (a+x—b+f+) donors) were absorbed in a similar way. Comparable results were obtained with the anti-D sera specific for Gm(a), Gm(b) or Gm(f).

Following absorption of anti-antibody in serum 647 using the reference anti-D sera, no reaction was detected even by anti CD Ripley. In contrast, after a similar absorption serum 576 still agglutinated red cells sensitized by anti-CD Ripley, but this reaction was not of the anti-antibody type (see later). Thus, only anti-antibody molecules of one specificity were detected in the sera 55, 576 and 647.

Absorption of Anti-Gm(f)

As the present and previous investigations (18) showed that the activity of Type I anti-antibodies was related to Gm(f) anti D absorp-

tion of anti Gm(f) was included (Fig 1) Results similar to those with anti antibodies of Type I were obtained Red cells sensitized by Gm(b) anti D 3109 were probably the most sensitive indicator as they contained only minor amounts of Gm(f) molecules This experiment thus further supported that anti antibodies of Type I were directed against immune complexes containing Gm(f) γG globulin

Absorption of Anti Antibodies of Type II

When absorptions were performed with anti antibody 306 (from a Gm(a+x—b+f+) donor) red cells sensitized by Gm(a) anti D removed the corresponding activity while that detected by Gm(f) anti D was maintained (Table 2) Red cells sensitized by Gm(f) anti D removed the corresponding activity but not that demonstrable by Gm(a) anti D Agglutinating activities in absorbed sera were not inhibited by native γ globulin (see above) When both types of activity were removed by absorption no agglutination was detected with red cells sensitized by anti D 3173 or anti CD Ripley Accordingly serum 306 contained two types of anti antibody molecules with different serological specificity

TABLE 2

Titres of Anti Antibody Serum 306 (Type II) in Saline (A) and in One per Cent γ Globulin (B) before and after Absorption with Red Cells Sensitized by Gm(a) Anti D (3091) or by Gm(f) Anti D (3083)

		Absorption with red cells		
		unsensitized	sensitized by Gm(a) anti D	sensitized by Gm(f) anti D
Test with				
Gm(a) anti D	A	16	<2	16
	B	16	<2	16
Gm(f) anti D	A	8	8	<2
	B	8	8	<2

Controls Unsensitized red cells and serum 306 (1:2 to 1:32) no agglutination The respective sensitized red cells and saline or one per cent γ globulin no agglutination

Anti antibody 648 (from a Gm(a+x—b+f+) donor) was then absorbed A new sample of this serum showed a weak reaction with red cells sensitized by Gm(a) anti D in addition to the previously described reaction detected by Gm(f) anti D Red cells sensitized by Gm(a) anti D removed the corresponding activity while that detected by Gm(f) anti D was maintained Inhibition experiments with native γ globulin (see above) showed that this activity was still of the anti antibody type However red cells sensitized by Gm(f) anti D removed the activity detected both by Gm(a) and by Gm(f) anti D

In absorptions with anti antibody 640 (from a Gm(a+x—b—f) donor) results similar to those for serum 648 were obtained Sera

648 and 649 thus both contained at least 2 types of anti antibody molecules, one specific for red cells sensitized by Gm(f) anti-D, the other cross-reacting (specific for Gm(a) and for Gm(f) anti D sensitized red cells)

In serum Be (from a Gm(a+x+b—f—) donor) only cross-reacting anti antibody molecules were found

Gm(b) anti-D 3109 containing a minor amount of Gm(f) molecules was used for absorption of anti-antibody sera of Type II After 2 or 3 absorptions the results were similar to those obtained after one absorption with Gm(f) anti-D 3083 (cfr results designated in Fig 1 with anti-antibody of Type I)

Anti-antibody sera 648 and Be (but not serum 649) agglutinated red cells sensitized by anti-CD Ripley after the removal of all activity detected by the reference anti-D sera (see later).

Absorption of Anti Antibody of Type III

Anti-antibody serum 141 (from a Gm(a+x—b+f+) donor) was also absorbed with sensitized red cells carrying the Gm(a), Gm(b) or Gm(f) specificities In all absorptions the corresponding activity was removed while the activities detected with the other two systems were hardly influenced (Table 3) Similar results were obtained with red cells sensitized by the other Gm(a) or Gm(f) anti-D sera

Native γ-globulin did not inhibit any of the activities remaining in the supernates after absorptions (see above) Furthermore when the activities, detected by Gm(a), Gm(b) or Gm(f) anti-D were all removed no agglutination was obtained even by using anti-CD Ripley Accordingly 3 types of anti-antibody molecules were found in serum 141

TABLE 3

Titres of Anti Antibody Serum 141 (Type III) in Saline (A) and in One per Cent γ Globulin (B) before and after Absorption with Red Cells Sensitized by Gm(a) Anti D (3091) by Gm(b) Anti D (3109) or by Gm(f) Anti D (3083)

			Absorption with red cells		
		unsensitized	sensitized by Gm(a) anti D	sensitized by Gm(b) anti D*	sensitized by Gm(f) anti D
Test with					
Gm(a) anti D	A	32	<2	32	16
	B	32	<2	32	16
Gm(b) anti D	A	8	4	<2	4
	B	8	4	<2	4
Gm(f) anti D	A	32	16	16	<2
	B	32	16	16	<2

* For some properties of this anti D when used in repeated absorptions cfr Fig 1
Controls Unsensitized red cells and serum 141 (1 2 to 1 64) no agglutination
The respective sensitized red cells and saline or one per cent γ-globulin no agglutination

Anti γ-Globulin Factors "Masked" by Anti Antibodies

Three sera (576, 648 and Be) still agglutinated red cells sensitized by the polyspecific anti CD Ripley after the removal of anti antibody activity using both Gm(a) (and Gm(x)), Gm(b) and Gm(f) anti D. The "masked" anti γ globulin factors were thus not specific for Gm(a), Gm(x), Gm(b) or Gm(f) anti D.

Inhibition experiments demonstrated that the factors in sera 576 and 648 were inhibited completely by one per cent pooled native γ globulin while only partially by one per mille γ globulin. The corresponding factor in serum Be was only partially inhibited even by one per cent γ globulin. None of the 3 factors were inhibited by pepsin digested pooled native γ globulin. Results obtained with serum 576 are shown in Table 4.

TABLE 4

Titres of Anti Antibody Serum 576 in Saline (A), in One per Cent γ Globulin (B) and in 0.6 per Cent Pepsin Digested γ Globulin (C) before and after Absorptions with Red Cells Sensitized by Gm(a) Anti D (3091) or by Gm(f) Anti D (3083)

		Absorption with red cells		
		unsensitized	sensitized by Gm(a) anti D	sensitized by Gm(f) anti D
Test with				
	A	64	64	<2
Gm(f) anti D	B	32	64	<2
	C	64	64	<2
	A	32	32	32
Anti CD Ripley	B	32	32	<2
	C	32	32	32

Controls: Unsensitized red cells and serum 576 (1:2 to 1:128): no agglutination. The respective sensitized red cells and saline: no agglutination. Sensitized red cells and undigested or pepsin digested γ globulin: no agglutination.

DISCUSSION

Anti Gm factors with different specificities have previously been demonstrated in individual sera (3, 9, 20). An anti Gm and anti Inv factor (21) or an anti Gm factor and an anti antibody (18) could also coexist in one serum.

In all but one of the present sera anti antibody molecules with only one specificity (monospecific) were demonstrated. Such monospecific molecules were detected by reference Gm(a), Gm(b) or Gm(f) anti D respectively. By proper absorption procedures some polyspecific anti antibody sera could become monospecific. Accordingly anti antibodies with specificities comparable to those of anti Gm factors were demonstrated.

Two sera (648 and 649) contained both monospecific and cross reacting molecules and one serum only cross reacting ones. It is possible, however, that more selected absorption procedures might

reveal other monospecific anti antibody molecules In contrast, cross-reacting factors might also exist in the sera containing different monospecific molecules It is well established that the heterogeneity in serological reactions with other anti- γ-globulin factors is at least partly based on the existence of separate molecules with different specificities besides cross-reacting factor(s) (14, 15, 24) Some theoretical aspects of monospecific and cross-reacting antibodies were recently discussed (11)

Anti antibodies were detected by anti-D of a Gm specificity represented among the donors own γG globulins (18) The only exception was that anti-antibodies from Gm(f—) donors (649 and Be) showed specificity for Gm(f) anti D Accordingly the cross-reacting anti antibody molecules in these two sera might be related to the particular Gm type of the donors However, a cross-reacting factor was also found in one Gm(f+) anti-antibody serum (648) This serum further demonstrated that a change in anti-antibody specificities may take place in individual sera, just as for anti-Gm factors (see 20) Sera containing both mono and polyspecific anti-antibodies with other specificities than here described may be found A serum with a single anti antibody detected by Gm(a) anti-D but not by Gm(b) or Gm(f) anti D has recently been reported (4) Anti-antibodies of such a specificity was in the present material only demonstrated in absorbed sera (cfr Tables 2 and 3)

In 2 sera (576 and 648), absorbed using the reference Gm(a), Gm(b) and Gm(f) anti-D, agglutinating activity detected by anti-CD Ripley was completely inhibited by pooled native γ globulin but not by pepsin digested γ globulin (Fab fragments) These are properties of rheumatoid factors directed against determinants of the Fc part of the γG globulin molecules (see 1, 2, 6) The activities might correspond to Gm factors present in low concentration in the γ globulin pool as complete inhibition was not obtained with one per mille γ-globulin However, certain properties of the particular reaction may also account for these findings

The corresponding activity of the absorbed serum Be was only partially inhibited even by one per cent γ-globulin This serum might contain a mixture of a rheumatoid factor, and an anti-antibody of another specificity than those detected by the reference anti D sera Further investigations on these "masked" factors are needed

"Masked" anti Gm factors have been demonstrated by Podliachouk et al (20) These findings emphazises careful examination for anti γ-globulin factors using highly specific agglutination and absorption procedures

The present results explain why anti-antibodies suitable as substitutes for antiglobulin (Coombs') sera have not been found (4) Even the most polyspecific anti antibody serum presented here was not suited for this purpose, as it was less sensitive for Gm(b) anti D

(cfr 16, Table 3, 18) Furthermore, even the sera containing monospecific anti antibodies could not be used for ordinary Gm typing as they were not inhibited by native γ-globulin On the other hand, they may prove valuable for the typing of isolated antibodies in immune complexes

In sera from rheumatoid arthritis patients, anti-Gm(f) (5, 8) or anti Inv (21) factors have so far not been found These factors both react with determinants on the Fab fragments (1, 8, 12) In contrast, rheumatoid factors often found in such sera react with determinants on the Fc fragment of γG globulin (2, 6) All sera investigated (18) and a recently detected anti antibody (700), including sera from 6 patients with rheumatoid arthritis (donors of sera 55, 355, 576, 647, 648 and 700) contained anti-antibody activity detected by Gm(f) anti D Although the specificity was related to Gm(f) molecules the anti antibodies probably indicated other determinants than the Gm(f) site itself (18), and might involve the Fc fragment (10) Similar findings of antisera directed against different parts of the same molecules were reported for anti-Gm(f) and anti Gm(p) (12, 23) and with antisera raised in different species against heavy chains of VI subgroup of isolated myeloma protein (7)

Observations to be published indicated that human anti antibodies react with determinants on the Fab fragments of γG globulin

SUMMARY

1 Eight human anti-antibody sera were investigated The anti antibodies showed different specificities in reaction with red cells sensitized by incomplete anti D Individual sera contained one, two or three types of anti-antibodies directed against immune complexes containing Gm (a), Gm(b) or Gm(f) γG antibodies, respectively

2 Sera with only one type of anti antibody molecule all showed specificity for immune complexes with Gm(f) anti D Even such monospecific anti antibody sera were not suited for ordinary Gm typing

3 After absorption of anti antibody activity, "masked" anti-γ-globulin factors not showing specificity for Gm(a), Gm(x), Gm(b) or Gm(f) anti D were demonstrated in three sera

REFERENCES

1 *Deicher H Ropart C Rivat L & Rousseau P Y* Lokalisation einiger erblicher Immunoglobulingruppen Eigenschaften auf Bruchstucken normaler G Immunoglobuline Humangenetik *1* 374–378 1965

2 *Franklin E C* The interaction of rheumatoid factors and gamma globulins Proc 10th Int Congr Rheumatology Rome 1961 Torino Minerva medica 2 804 805 1961

3 *Fudenberg H H & Kunkel H G* Specificity of the reaction between rheumatoid factors and gamma globulin J exp Med *114* 257 278 1961

4 *Gold F R & Lockyer J R* • Production of two kinds of anti gamma• gl bulin in the rabbit and reactions of an anti antibody with D positive an l D– cells *Vox Sang. 9* 573–577, 1964
5 *Gold E R., Mårtensson L Ropart• C Rivat L & Rousseau P Y* Gm(f)—a determinant of human γ globulin Vox Sang *10* 299–302, 1965
6 *Goodman J W* Reaction of rheumatoid sera with fragments of papain digested rabbit gamma globulin Proc Soc exp Biol (N Y) *106* 822 82, 1961
7 *Grey H M & Kunkel H G* H chain subgroups of myeloma proteins and normal 7S γ-globulin J exp Med *120* 253 266, 1964
8 *Grubb R Kronvall G & Mårtensson L* Some aspects of the relations between rheumatoid arthritis antigamma globulin factors and the polymorphism of human gamma globulin Ann N Y Acad Sci *124* 865–872 1965
9 *Harboe M Simultaneous occurrence of hemagglutinating substances of different* specificities in rheumatoid sera Acta path et microbiol scandinav 49 381 392 1960
10 *Harboe M Rau B & Aho K* Properties of various anti γ globulin factors in human sera J exp Med *121* 503 519 1965
11 *Hirschfel l J Serologic codes interpretation of immunogenetic systems* Science *148* 968–971 1965
12 *Kronvall G* Gm(f) activity of human gamma globulin fragments Vox Sang *10* 303 313 1965
13 *Lowry O H Rosenbrough N J., Farr A L & Randall R J* Protein measurement with the Folin phenol reagent J biol Chem *193* 265–275, 1951
14 *Milgrom F & Tonder O* Multiplicity of rheumatoid factor Arthr and Rheum 8 203 212 1965
15 *Milgrom F Witebsky E Goldstein R & Loza U* Studies on the rheumatoid and related serum factors J Amer med Ass *181* 476–484 1962
16 *Natvig J B* Incomplete anti D antibody with changed Gm specificity Acta path et microbiol scandinav *65* 467–479 1965
17 *Natvig J B* Quantitative determinations of incomplete anti D antibodies in reaction with anti Gm factors Acta path et microbiol scandinav *65* 570 580 1965
18 *Natvig J B* Specificity of anti antibodies in human sera Acta path et micro biol scandinav *65* 559 569, 1965
19 *Osterland C K Harboe M & Kunkel H G* Anti γ globulin factors in human sera revealed by enzymatic splitting of anti Rh antibodies Vox Sang *8* 133 152 1963
20 *Podliachouk L Eyquem A & Jacqueline F* Observations sur la coexistence des substances anti Gm(a) et anti-Gm(x) et sur le choix des réactifs anti Gm(b) Ann Inst. Pasteur *107* 280–285 1964
21 *Ropart• C Vierucci A Rivat L & Rousseau P Y* *Présence simultanée d'un* anti Gm et d'un anti Inv chez un polytransfusé Rev franç Etud clin biol 9 977 978 1964
22 *Waller M V Decker B Toone E C & Irby R* Evaluation of rheumat id factor tests Arthr and Rheum 4 579 591 1961
23 *Waller M Hughes R D Townsend J I Franklin E C & Fudenberg H* New serum group Gm(p) Science *142* 1321 1322 1963
24 *Williams R C & Kunkel H G* Separati n of rheumatoid factors f different specificities using columns conjugated with gamma gl bulin Arthr and Rheum 6 665–675 1963

Acta path et microbiol scandinav 66 197 206 1966

The National Institute of Public Health Virus Department A Oslo Norway

PRODUCTION OF POLYOMA VIRUS HAEMAGGLUTINATION INHIBITORS IN MOUSE KIDNEY EPITHELIAL MONOLAYERS

By

GUNNAR HAUKENES and O LAHELLE

Received 20 viii 65

Propagation of polyoma virus is routinely obtained through the use of mouse embryo monolayers Growth of the virus has been reported to occur also in other tissues and other species (4 10, 11) *Winocour* (11) produced high titre polyoma virus pools in kidney cultures originating from *in vivo* polyoma inoculated suckling mice Mouse kidney cultures prepared from non inoculated 12 to 15 day-old mice and infected *in vitro* gave lower virus titres than *in vivo* infected cultures The titres obtained by *in vitro* infection were however, considerably higher than those obtained by the ordinary mouse embryo culture method The mouse kidney cultures were predominantly epithelial like and a strong cytopathic effect was observed in polyoma infected cultures

Also *Orth et al* (10) have reported high titres of polyoma virus in mouse kidney cultures

The present work started with the intention to prepare pure epithelial mouse kidney monolayers for polyoma virus experiments It soon became evident that mouse kidney cultures made from a dispersed kidney mince and treated in various ways could not be obtained completely free of fibroblastic cells It was observed however that these cultures had high contents of a temperature dependent polyoma virus haemagglutination inhibitor which will be reported in some detail below

MATERIALS AND METHODS

Virus The SE polyoma virus strain originated from Sara Stewart s strain It was obtained from Dr *H R Morgan* University of Rochester N Y and had been through several passages in mouse embryo cultures in his and this laboratory The Toronto polyoma strain was received from Dr *J C Ulstrup* Oslo City Hospital and originated from the laboratory of Dr *M G P Stoker* University of Glasgow No differences between the strains were observed in the present study with regard to haemagglutination inhibition Unless otherwise stated the SE polyoma strain has been employed

Cells and media All mice used for tissue culture were random bred Swiss albinos. Mouse embryo (MF) cultures were prepared by the usual method by trypsin digestion of minced embryos. *Mouse kidney epithelial* (MkE) cultures were prepared from mice of different ages from new born to 23 days old. The kidneys were removed aseptically and minced into small pieces by scissors followed by repeated washings. The mince was thereafter incubated with 0.1 per cent trypsin (Difco 1 250) and 0.01 per cent collagenase (Worthington Biochemical Corporation) at 37° C for 30 minutes under constant stirring. The tissue fragments were then allowed to settle and the supernatant fluid decanted. This procedure was repeated twice whereupon the residual tissue fragments were suspended in the culture medium without serum and shaken by hand. All four supernates were combined and transferred to Roux bottles, Petri dishes or tubes for cultivation.

The cloning experiments were carried out by the micro drop method (9).

Eagle's basal medium supplemented with serum, penicillin and streptomycin was used in all cultures. For primary growth 15 per cent calf serum or bovine foetal serum was added and after infection 4 per cent horse serum. In the micro-drop medium the amino acid and vitamin contents were doubled.

Infection The cultures were infected from one great pool of virus yielding a haemagglutination titre of 1 128 to 1 256. In accordance with newer findings (8) incubation with virus was carried out at 4° C or at room temperature for one hour whereupon the medium was added and incubated at 37° C.

Haemagglutination (HA) assays The withdrawn culture fluid samples to be

speed to remove

° C for 30 minutes

receptor destroying

ried out at 37° C

overnight followed by heating at 56° C for one hour.

The HA was performed in tubes by mixing equal volumes of two fold serial dilutions of virus in phosphate buffered saline (PBS) of pH 7.2 and 0.4 per cent guinea pig red blood cells, 0.2 ml of each. The red blood cells were suspended in PBS containing 10 per cent of a 30 per cent bovine serum albumin solution (Armour Pharmaceutical Company). HA readings were made after overnight incubation at 4° C. When direct comparisons of HA titres are reported below all titrations have been carried out with the same erythrocyte suspension.

EXPERIMENTAL AND RESULTS

Characteristics of MkE Cultures

A number of cultures were made from mice of varying ages and 6 to 9 days old mice seemed to give the most satisfactory results. When cultures were prepared from older mice the growth was often more scarce and varying. Mice younger than 6 days gave rise to cultures with more fibroblasts and the cultures were almost entirely fibroblastic when made from new born mice.

A confluent monolayer usually formed after 3 to 5 days of incubation and the cells were evidently epithelial like. On further incubation however fibroblastic foci with outgrowing stripes could be observed in some cultures. It seemed to be of importance to ensure a rapid outgrowth of a confluent epithelial monolayer to suppress growth of fibroblasts, one possible reason why 6 to 9 day old mice gave best results. Therefore also a heavy inoculum of cells was used. As a consequence the cultures needed regular checking for fibrocellular contaminations and when a culture is referred to here as epithelial no or only scattered fibroblastic elements were observed.

All attempts at cloning the epithelial cells were unsuccessful Some single cells multiplied, giving rise to small colonies of 10 to 30 cells, whereupon the cells degenerated

Polyoma Virus Infected MKE Cultures

Virus haemagglutinins usually appeared in the culture fluid 3 to 5 days after infection of MKE cultures with polyoma virus The HA titre reached a maximum after an additional two or three days of incubation and remained at this level for about three weeks While ME cultures seldom gave HA titres above 1 256, titres as high as 1 2048 or 1 4096 were frequently encountered in MKE cultures

In some MKE cultures high titres of virus were obtained without visible changes of the cells, but further incubation usually resulted in cell degeneration However, very often some degree of degeneration also occurred in the non-infected controls, thus rendering the evaluation of cytopathic effects uncertain

HA Inhibitors in MKE Cultures

The HA test was in the beginning carried out as noted under "Methods' including centrifugation, heat inactivation and serial dilution of the sample, addition of guinea pig red cells and incubation in the cold However, preliminary trials with heat inactivated virus preparations frequently showed great variations in regard to HA Thus the same sample examined with one day interval might drop from 1 2048 to 1 16 After treatment of the virus samples with RDE, maximal and reproducible titres were obtained (cf Table 1) This did indicate the presence of a temperature dependent HA inhibitor like that described in ME cultures (6) Since the HA procedures were the same in our ML cultures which gave reproducible results, it seemed to be of some interest to study the inhibitors of the MKE cultures in some detail

The HA inhibition of the MKE culture reported above is most likely due to a lowering of the temperature during the dilution procedure Table 1 shows the HA patterns of a polyoma infected MKE culture fluid when the HA test was carried out with or without heating of the virus preparations at 37° C for 30 minutes before addition of red blood cells, compared with an RDE treated sample An MF culture, treated in the same way, has also been included in the table It is apparent that the HA is strongly inhibited in the MKE culture when the temperature is allowed to drop before addition of the erythrocytes, and this inhibition is almost completely abolished by heating of the sample at 37° C The HA titre of the ME culture, on the other hand, is not influenced by pre-heating

Portions of infected culture fluid were then incubated at 4°, 20° and 37° C for 30 minutes as shown in Table 2 One ME culture fluid has been included for comparison

TABLE 1

HA Titres of Polyoma Infected MKE and ME Culture Fluids with and without Pre Heating at 37° C for 30 Minutes before Addition of Red Blood Cells Compared with RDE Treated Samples

Sample	Pre treatment	HA in dilution (reciprocal values) 16	32	64	128	256	512	1024	2048
MKE (V9469)	No treatment	—	—	—	—	—	—	—	—
	37° C 30 minutes	+++	+++	+++	+++	+++	+	—	—
	RDE	+++	+++	+++	+++	+++	+++	(+)	—
ME (V9467)	No treatment	+++	+++	+++	—	—	—		
	37° C 30 minutes	+++	+++	+++	—	—	—		
	RDE	+++	+++	+++	+++	+++	—		

(+) to +++ varying degrees of haemagglutination
— no haemagglutination

The results obtained in this experiment and additional experiments were just about the same. The MKE culture fluids regularly inhibited HA by pre-incubation in the cold, while ME fluids showed no temperature dependent inhibition. Both cultures gave higher titres after RDE treatment. The inhibition in the cold was reversible as shown in Table 2, since the virus haemagglutinin was unmasked by heating of the reagents after overnight incubation in the cold.

TABLE 2

HA of Polyoma Infected Cultures Pre-Heated at Various Temperatures before Addition of Red Blood Cells

Sample	Treatment	HA titre
MKE (V 9469)	4° C 30 min	<1:16
"	20° C 30 min.	<1:16
"	37° C 30 min	1:512
	RDE	1:1024
"	4° C 30 min and 37° C 30 min	1:512*
ME (V 9467)	4° C 30 min	1:64
"	20° C 30 min	1:64
"	37° C 30 min	1:64
	RDE	1:256

After incubation at 4° C the red cells were added and the tubes left overnight in the cold without giving haemagglutination in the dilution 1:16. The tubes were then shaken, incubated at 37° C for 30 minutes and the haemagglutination read after incubation in the cold.

TABLE 3

Influence on HA Titre by Pre-Incubation of Polyoma Infected MKE Culture Fluids in the Cold before and after Treatment with RDE

Sample	Treatment of sample before addition of erythrocytes	HA titre
MKE (V 9756)	4° C 30 min	<1:2
"	37° C 30 min	1:128
"	RDE and subsequently 4° C 30 min	1:256
	RDE and subsequently 37° C 30 min	1:256

Usually incubation in the cold gave a more complete inhibition than at 20° C. Under both conditions inhibition sometimes occured only at low dilution (cf. the HA patterns in Table 5).

As might be expected no inhibition could be obtained by incubation in the cold after treatment of the virus sample with RDE as shown in Table 3.

TABLE 1

HA Titres of Polyoma Infected MKF and MF Culture Fluids with and without Pre-Heating at 37° C for 30 Minutes before Addition of Red Blood Cells Compared with RDF Treated Samples

Sample	Pre treatment	HA in dilution (reciprocal values)							
		16	32	64	128	256	512	1024	2048
MKF(V 9469)	No treatment	—	—	-	—	—	—	—	—
„	37° C 30 minutes	+++	+++	+++	+++	+++	+	—	—
„	RDF	+++	+++	+++	+++	+++	+++	(+)	—
MF(V 9467)	No treatment	+++	+++	+++	—	—	—		
„	37° C 30 minutes	+++	+++	+++	—	—	—		
„	RDF	+++	+++	+++	+++	+++	—		

(+) to +++ varying degrees of haemagglutination
-- no haemagglutination

In one experiment fibroblast cultures were prepared from the same mice as used for MKE cultures These fibroblast cultures behaved like the ME cultures with regard to temperature dependent inhibitors, not being inhibited by incubation in the cold

It was of some interest to see whether the inhibitors were released as a result of virus infection of the cells Supernatant fluids of infected and non-infected cultures were tested for HA inhibition in the haem agglutination inhibition (HI) test against 8 HA doses per tube of RDE treated virus The fluid samples were incubated with the test virus at 4°, 20° or 37° C for 60 minutes before addition of red blood cells and incubation overnight in the cold (Table 4) The culture fluids to be tested were heated at 72° C for 30 minutes in advance At this temperature the virus haemagglutinins are destroyed without demonstrable influence on the inhibitors concerned

Incubation at 20° C (not included in the table) gave almost the same HI patterns as 4° C It is seen from Table 4 that infected and non-infected cultures showed almost the same degree of HA inhibition It is evident that some inhibition also occurred by incubation at 37° C ME cultures showed some HA inhibition when the test was carried out in this way

HA inhibition was not dependent on the source of virus Thus virus from ME monolayers and MKE cultures was inhibited equally well No difference between the SE and the Toronto strain of virus was recorded with regard to HA inhibition

As mentioned under "Methods" the supernatant culture fluids were routinely centrifuged at low speed (about 2000 r p m) to remove whole cells and cell fragments If the temperature was allowed to drop to room temperature (20 to 21° C) before and during the centrifugation, the HA titres were greatly reduced, *e g* from 1 1024 to 1 64 in one experiment Centrifugation at room temperature may thus remove great quantities of virus

In another experiment the centrifugation was carried out at 37° C, and the inhibitory capacity of the fluids tested after centrifugation After centrifugation at 2000 r p m and 9000 r p m the fluids still contained some inhibitors, and in this experiment the retained inhibition appeared to be active only at low dilutions of the fluids (cf Table 5)

DISCUSSION

When prepared from young mice, preferentially 6 to 9 days old, mouse kidney epithelial cultures could be obtained almost free of fibroblastic contamination Since the establishment of a clonal line of the epithelial cells was not successful, further studies of polyoma virus-induced changes of the cells were not performed In accordance with *Winocour's* (11) findings our MKE cultures have HA titres up to 20 times higher than those obtained with ME cultures

TABLE 5

HA Patterns of Polyoma Infected Culture Fluids before and after Centrifugation at 2000 r p m and 9000 r p m

Culture fluid	Centrifugation at 37° C	Incubation temperature before addition of red cells	HA in dilution (reciprocal values) 4	8	16	32	64	128	256	512	1024	2048
MKI (A 9469)	No centrifugation	4° C	—	—	—	—	—	—	—	—	+	—
	2000 r p m 10 min	4° C	—	—	—	—	—	+++	+++	++	++	—
	9000 r p m 10 min	4° C		—	—	+	+	++	++	+	—	—
	No centrifugation	37° C	+++	+++	+++	+++	+++	+++	+++	++	—	—
	2000 r p m 10 min	37° C	+++	+++	+++	+++	+++	+++	++	++	+	—
	9000 r p m 10 min	37° C	+++	+++	+++	+++	+++	+++	+++	+	—	—

The studies of the temperature dependent HA inhibitor demonstrate the importance of strict procedures in polyoma virus yield assays especially when testing newer cultivation methods A drop to room temperature before and during centrifugation removes considerable quantities of virus and the HA titres will be misleading unless warming at 37° C is performed prior to addition of red cells if the samples are not treated with RDE It is also evident that it is not safe to perform screening HA tests with only one dilution of the sample since the inhibitors to some extent are active only at certain dilutions

The centrifugation experiments at 37° C (cf Table 5) may indicate that at least some of the temperature dependent inhibitory capacity is not connected to cells cell fragments or greater sub particular elements *Dahl et al* (3) examining the inhibition of polyoma HA by cell fractions of P 388 D1 and Detroit 6 cells found highest inhibitor concentrations in the mitochondrial fraction and in the cell sap

Extracts of mouse kidney tissue have been found to have high contents of polyoma virus HA inhibitors (2 7) *Baldux i & Salmon* (2) found that mouse organ extracts inhibited the polyoma virus HA both at 37° and 4° C in contrast to serum inhibitors which inhibited only at 4° C *Baldu i* (1) also found that the unmasking of polyoma virus haemagglutinin by heating and the masking by storage at 4° C were more rapid with red cell eluates than with crude virus from polyoma infected culture fluid He concludes that this suggests the presence of more than one inhibitor in the crude virus preparation

Most likely the inhibitors described here are the same as the ones reported by *Deinhardt et al* (6) as being present in MKE cultures They recommend pre heating at 37° C as above to allow maximal release of virus before adding red cells However this procedure does not seem to have been uniformly adopted and in contrast to their findings preincubation at 37° C had little influence on the HA titres in MKE cultures Strain line differences as reported by *Diamond & Crawford* (5) are a possible explanation of this discrepancy

SUMMARY

Preparation of a mouse kidney epithelial culture completely free of fibroblastic elements was not successful The MKE culture gave high yields of polyoma virus

The temperature dependent inhibitor described by *Deinhardt et al* (6) was found to interfere greatly with the haemagglutination of virus from MKE cultures and special measures to avoid this are outlined

REFERENCES

1 *Baldu* P L emoagglutinazione da v rus S F pol oma Lo sperimentale *111* 247 263 1961

2 *Baldu* P & *Salmon* S Inh bitori della emoagglutinaz one da virus pol oma in sieri e organi di topi normali Lo sper mentale *112* 446–456 1962

3 *Dahl D Jonsen J & Lahelle O* Study of the cell bound inhibitor of polyoma virus hemagglutination Acta path et microbiol scandinav In press
4 *Dawe C J* Cell sensitivity and specificity of response to polyoma virus J Natl Cancer Instit Monogr no 4 p 67 125 1960
5 *Diamond L & Crawford L V* Some characteristics of large plaque and small plaque lines of polyoma virus Virology 22 235-244 1964
6 *Deinhardt F Henle G & Marks M* The effect of inhibitors on the haemagglutinating activity of polyoma virus J Immunol 84 559 607 1960
7 *Diderholm H & Wesslen T* Interaction between polyoma virus and different tissues of mouse and hamster *in vitro* Archiv gesamt Virusforsch 14 45-54 1963
8 *Helgeland K Lahelle O & Jonsen J* Polyoma virus 1 Cell virus interaction at low temperature Acta path et microbiol scandinav 58 511 516 1963
9 *Lwoff A Dulbecco R Vogt M & Lwoff M* Kinetics of the release of polio myelitis virus from single cells Virology 1 128 139 1955
10 *Orth G Atanasiu P Lamonthezie N & Paoletti C* Présence d inclusions spécifiques dans les cultures de cellules renales de souriceau inoculées avec le virus du polyome ou son acide nucleique Annal Instit Pasteur 103 657 664 1962
11 *Winocour E* Purification of polyoma virus Virology 19 158 168 1963

Acta path et microbiol scandinav 66 207-226, 1966

The Department of Medical Microbiology, Uppsala University, Uppsala, Sweden

THE ETIOLOGY OF RESPIRATORY TRACT INFECTIONS IN MILITARY PERSONNEL

6 *A Critical Evaluation of a Study during the Years 1960–1963*

By

R Berg, G Lindbom, G Laurell, G Löfström, L Philipson and T Wesslén

Received 25 viii 65

The syndrome of upper respiratory tract infection is still open to discussion from an etiological point of view but it is generally accepted that as many as 100 different viruses may cause the various types of disease The present situation has been fully covered in a recent conference on Newer Respiratory Viruses (1) Data are available concerning a number of new respiratory viruses but they permit us to speak merely of the existence of these biological species and their properties rather than of their role in human pathology

In previous communications (2–6) bacteriological and virological studies for the years 1959–1960 on acute respiratory infections among military personnel in Sweden were presented The incidence of illness was low and attempts to correlate the clinical and etiological findings were discouraging A probable etiology was, however, established in about 66 per cent of infections, 52 per cent virus infections and 14 per cent bacterial infections mainly by hemolytic streptococci Adeno- and influenza viruses were considered responsible for the majority of the virus infections identified

The present study reports the results of an additional three year period

Plan of the Study

The investigation was carried out at a military camp at Uppsala The number of conscripts varied considerably—between 700 and 1698—partly due to the fact that the conscripts were on duty elsewhere in Sweden for various length of time, when they could not be examined

All conscripts, reporting for respiratory tract infections at the hospital of the camp, were examined daily by one physician and one assistant from the laboratory Disease history and symptoms were registered During the autumn of 1962 and the winter of 1963 controls were included in the study They were collected at three occasions, two

at an interval of one month in the autumn 1962 and one in february 1963 The following specimens were taken at the various visits

Specimens from the nose and throat for bacteriological and virological examinations were taken at the first visit and five days later

Faeces specimens were taken for virological examination at the same intervals

Blood samples were collected at the first visit and repeated twice at ten-day intervals

The course of the disease was recorded each time a specimen was taken The whole study was carried out in close collaboration with the head physician of the establishment

MATERIAL AND METHODS

Collection of Specimens

Throat The methods were described previously (2 5, 6) The only change undertaken is that the 5 per cent horse serum in the Parker 199 medium was substituted with 0 5 per cent of gelatin

Faeces and blood specimens were handled as described before (2, 5 6)

Tissue Cultures

Monkey kidney Trypsinization of Rhesus or Cynomolgus monkey kidney was carried out according to methods described before (2) The maintenance medium was Eagles minimal essential medium (MEM) (7) with antibiotics (100 units and 100 mcg/ml of penicillin and streptomycin respectively)

Human kidney or human lung Tissues from embryos delivered from abortion and from premature infants were trypsinized by the ordinary methods for monkey kidney (8) or in the cold according to methods described (9) The maintenance medium of these cultures was Eagles MEM with antibiotics

Hemadsorption Test

The hemadsorption test was carried out according to the modification of *Vogel & Shelokov* s technique (10) reported by *Chanock et al* (11)

Virus Isolations

All virological specimens from throat and faeces were inoculated on the various tissue cultures monkey kidney human kidney or human lung according to the methods described before (2 5 6) Incubation at both 33° C and 37° C of the cultures were performed

Isolation of Bacteria

The specimens were examined according to techniques previously described (3) Potential pathogens of the respiratory tract as *Staphylococcus aureus* haemolytic streptococci *Haemophilus influenzae* pneumococci and meningococci were recorded The streptococci and pneumococci were typed as previously described (3)

Antibody Analysis

Complement fixation tests were carried out in tubes with 0 1 ml of antigen and serum respectively according to a method previously described (2) All sera from the diseased conscripts and controls were examined for complement fixing (CF) antibodies against adenovirus influenza A B and C viruses mumps and parainfluenza virus type 1 3, Herpes simplex ornithosis and ECHO 28 viruses In addition the [illegible]

preparation of most antigens was described in a previous communication (2) ECHO 28 CF antigen was prepared according to *Mogabgab* (12)

Antibacterial antibodies antistaphylolysin antistreptolysin and antipneumolysin were determined according to methods previously described (3) Complement fixing antibodies against H influenzae were determined with a sonicated extract of whole bacteria as the antigen

Definitions

In previous papers a fourfold increase in antibody titre was considered as an

(5 6)

Thus a virus may be considered as *a cause of infection* when a fourfold rise in antibody titre occurs during a certain period of time It cannot however be accepted as *the cause of a particular disease* or *an outbreak of disease* unless the rate of positive findings are significantly higher in sick persons than in healthy controls of the same population

spite of insufficient serological response The reason for this might be that an extensive antibiotic therapy was instituted at an early stage of the disease

Clinical Syndromes

The respiratory diseases observed were classified in four syndromes exudative pharyngitis (*ExP*) pharyngoconjunctivitis (*PC*) acute respiratory disease (ARD) and minor respiratory illness (MRI) according to the criteria of *Grieble et al* (13) and described in previous reports from this laboratory (4)

RESULTS

Total Incidence of Disease

All conscripts reporting for acute respiratory disease at the hospital of the military camp were examined

Fig 1 shows that the total incidence of disease in this population was comparatively low ranging from 0 3 per cent (Dec 1960) to 12 6 per cent (1st week of March 1963) of the total number of conscripts stationed at the camp

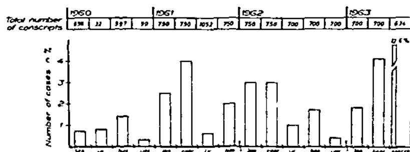


Fig 1

The incidence of disease in the conscripts during the entire period 1960 1963

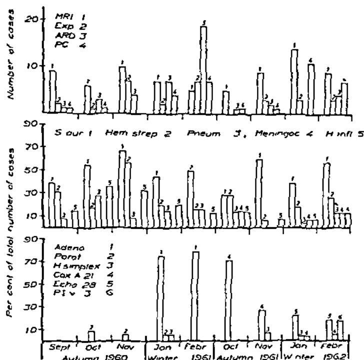


Fig 2

Distribution of clinical syndromes in comparison with the isolation of bacteria and significant increase in antibodies against viruses in sick conscripts of the years 1960–1962 The frequencies are given in per cent of total number of cases per month

The upper part of Figs 2 and 3 shows the distribution of the various clinical syndromes at monthly intervals during the investigation period 1960–1963

The middle part of the figure gives the potential bacterial pathogens isolated from the first swabs in per cent of the number of cases per month The lower part records the monthly frequency of cases showing a significant increase in antibody titres for the various species of viruses in per cent of the total number of cases for each month

Fig 3 also shows the results of examinations of healthy controls which was undertaken during the autumn of 1962 and the winter of 1963

The bacteriological and virological findings for the various periods are given below

Autumn 1960

A total of 45 sick conscripts were examined during this period

Virological findings Adenovirus type 4 was isolated from 1 case with constant high titres against adenovirus Three additional cases showed a significant increase in complement-fixing (CF) antibodies towards adenovirus between the first and the third serum sample Two cases

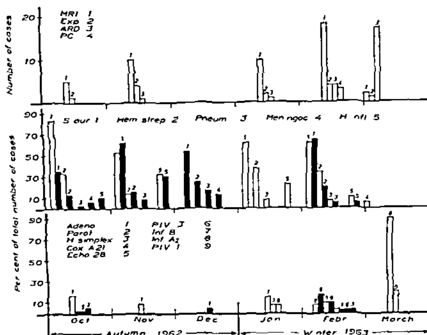


Fig 3

Distribution of clinical syndromes in comparison with the isolation of bacteria and significant increase in antibodies against viruses in sick conscripts and controls of the years 1962–1963 The frequencies are given in per cent of total number of cases per month Filled columns controls Unfilled columns sick conscripts

had a significant increase against parotitis virus and one case showed high titres in the Paul-Bunnell reaction

Bacteriological findings Hemolytic streptococci were isolated from 18 cases but only 2 showed an increase in antistreptolysin titre The combined clinical and bacteriological findings suggested an etiological role in a total of 5 cases The isolated strains from these cases belonged to group A type 22 in 3 cases and group C in one case One strain was untypable A concurrent adenovirus infection was observed in one case

H Influenzae was isolated in 13 cases, Staph aureus in 25 cases and pneumococci in 5 cases No serological or clinical evidence favoured an etiological role of these bacteria

Winter 1961

A total of 58 sick conscripts were examined during this period

Virological findings This investigation period was dominated by adenovirus type 4 In a total of 45 cases adenovirus type 4 was isolated and 42 of these cases showed a significant increase in CF antibodies Two of the cases showed constant high titres against adenovirus and in the last case a third serum sample was missing Three additional cases

had CF antibody increase without virus isolation One case had a secondary infection of adenovirus, showing a significant antibody titre increase between the second and the third serum sample Another case showed a significant increase in antibodies against parotitis virus and Herpes simplex virus was recovered from one case, which also had a significant antibody rise against this virus

Bacteriological findings Hemolytic streptococci were isolated from 10 cases, but only one case had a significant increase in antistreptolysin titre H influenzae was isolated in 7 cases, pneumococci in 9 and *Staph aureus* in 26 cases No cases were observed with significant increase in antibody titres

Autumn 1961

A total of 22 sick conscripts were examined during this period

Virological findings Coxsackie virus type A 21 was isolated from the throat samples in six cases, all with a significant antibody increase in both neutralization and hemagglutination—inhibition tests Two additional cases showed a significant antibody increase without the isolation of this virus In one case significant increase in neutralizing antibodies was found together with constant high titres of HI antibodies Herpes simplex virus was isolated from one case, which also had a significant antibody increase in the CF-test One case had a secondary adenovirus type 2 infection This outbreak of Coxsackie virus type A 21 infection has been reported in detail elsewhere (5)

Bacteriological findings Hemolytic streptococci were isolated in 3 cases, of which none had a significant increase in antistreptolysin

H influenzae was recovered from 2 cases, *Staph aureus* from 11 and pneumococci from a single case One case showed a significant increase in antistreptolysin without isolation of hemolytic streptococci

Winter 1962

A total of 51 sick conscripts was examined during this period

Virological findings ECHO virus type 28 was isolated in 2 cases and both had significant antibody rise in neutralization and CF tests Ten additional cases showed significant antibody rises although no virus was isolated Furthermore 15 cases had high titres against ECHO 28 in all three serum samples This outbreak of ECHO 28 infection was reported earlier (6) Adenovirus type 2 was isolated from two cases, one with high titres, the other with significant antibody increase in the two first serum samples The first case had a concurrent ECHO 28 infection One strain of parainfluenza type 3 was isolated from 1 case and 3 additional cases showed a significant rise in antibodies against this virus between the two first serum samples During this period two cases of Coxsackie A 21 infection were observed, one of them with a concurrent infection of parainfluenza virus type 3

Bacteriological findings Hemolytic streptococci were isolated from 10 cases but were considered of etiological importance in 2 of these cases only One was an exudative pharyngitis with heavy growth of streptococci of group A type 12 but no significant increase in anti streptolysin titre could be demonstrated The other conscript had a pharyngoconjunctivitis and hemolytic streptococci group G type 16 with an increase in antistreptolysin titre from 400 to 700 units between the two serum samples

H Influenzae was isolated from 5 cases pneumococci from 4 and *Staph aureus* from 24 sick conscripts

Autumn 1962

During this period a total of 21 sick conscripts and 98 controls were examined

Virological findings Adenovirus type 4 was isolated in one sick con script and a total of 2 cases in the sick group had a significant antibody increase against this virus In the controls adenovirus type 4 was iso lated from 2 cases both had a significant antibody increase against this virus An antibody increase against ECHO 28 was also observed in one single case among the controls Two controls also had significant anti body increase towards Herpes simplex

Bacteriological findings Hemolytic streptococci were isolated from 4 sick conscripts (18 2 per cent) compared with 19 cases in the controls (19 4 per cent) In one sick conscript the hemolytic streptococci were considered to be etiologically associated to the exudative pharyngitis observed Pneumococci and meningococci were less frequent among the sick conscripts than in the control group *Staph aureus* was found in the same frequency in the two groups *H influenzae* was recovered more often from sick than control cases but serological evidence of infection was not found in any case

Winter 1963

A total of 62 sick conscripts and 108 controls were examined during this period

Virological findings This investigation period was dominated by the outbreak of influenza type A 2 infection occurring at the termination of the study A single strain of the virus was isolated but 21 cases had serological evidence of infection among the sick conscripts and 19 among the controls Double infection with parainfluenza virus type 1 were likely in 4 diseased and 2 controls Serological evidence of single infections with parainfluenza type 1 was obtained from 2 diseased and 8 controls Adenovirus type 4 was isolated in 2 sick conscripts and a significant antibody increase was found in an additional case compared with 2 cases of serological conversion against adenovirus in the controls

Finally 1 sick and 2 control cases showed serologic evidence of infection with Herpes simplex virus

Bacteriological findings No secondary bacterial infections were observed among the sick conscripts with evidence of influenza virus infection Hemolytic streptococci were isolated in 24 2 per cent of the diseased and 20 4 per cent of the controls Only in 2 cases of exudative pharyngitis were the streptococci considered to be of etiological importance One cases showed abundance of streptococci group A type 22 and the other had a *significant increase of antistreptolysins* The other bacterial species were isolated in the same frequencies in both controls and diseased except for *H influenzae* which was present in 9 7 per cent of the sick conscripts against 4 6 per cent in the controls

Etiological Significance of the Microbial Findings

Conclusions based on the definitions given earlier in this paper can only be drawn from the periods during which control personnel was included in the study, starting in the autumn of 1962 The morbidity during the autumn was low, not exceeding 2 0 per cent The dominating clinical syndrome was MRI Only some adenovirus infections were of importance The frequency rate of type 4 infections was 9 5 per cent among the diseased and 2 per cent among the controls (χ^2 = 1 2 P > 0 05) According to the definitions the adenovirus infections may not be assigned etiological significance In total only 14 per cent of the diseases could be associated with the pathogens observed No definite etiological significance could however be proved statistically

During the winter months of 1963 the morbidity rate was highest in February (4 2 per cent) and during the first week of March (12 6 per cent calculated as the frequency rate per month) Influenza A 2 dominated and the frequency rate figures for the period 19 2 7 3 were for sick conscripts 69 per cent and for the controls 30 9 per cent of the total number of cases The difference is significant (χ 11 1 P > 0 001) The parainfluenza type 1 infections and the adenovirus type 4 infections which occurred in 9 6 and 4 8 per cent respectively in the sick conscripts and in 8 4 and 1 5 per cent respectively in the controls could on the other hand not with certainty be assigned any etiological significance In no case were the isolated bacteria of etiological significance in accordance to the definitions

In the periods in which no controls were included some observations on the etiological significance of the isolated pathogens may be mentioned

In the autumn 1960 only 24 per cent of the cases were associated with the pathogens under study and the etiology of most cases was uncertain Hemolytic streptococci were tentatively responsible for five infections adenovirus type 4 for three and parotitis virus for two infections

During the winter of 1961 adenovirus type 4 was most probably responsible for a total of 45 out of 58 cases One case was ascribed to each of the following pathogens hemolytic streptococci parotitis virus and Herpes simplex virus Thus 82 7 per cent of the observed cases could be associated with the pathogens under study

An outbreak of fairly uniform infections during the autumn of 1961 was most likely caused by Coxsackie type A 21 virus Nine of the 22 cases which occurred during this period belonged to this outbreak One additional case was probably caused by hemolytic streptococci It appeared that 50 per cent of the total number of cases was associated with the pathogens under study

Twelve of the observed 51 cases during the winter of 1962 could possibly be ascribed to an outbreak of ECHO type 28 virus Adenovirus type 2 may have been responsible for 2 cases parainfluenza virus type 3 for four hemolytic streptococci for two cases and Coxsackie type A 21 virus and influenza B virus for one case each Thus 43 per cent of the diseases observed could be associated with the pathogens under study

A total of 258 sick conscripts were examined during the period 1960 1962 and in 111 cases (42 9 per cent) the disease could be associated with an infection with one or more of the pathogens studied Of the virus infections the influenza A virus only fulfilled the criteria of the definitions of a pathogen as an etiological agent

Latent Infections

The serological surveys of the controls revealed that infection with adenovirus had occurred between the sampling in the autumn 1962 and the winter 1963 The same population was stationed at the camp during the entire year 1962 1963

Fig 4 shows that a major fraction of the controls had converted to higher titres of adenovirus complement fixing antibodies in their first blood samples between the two periods The sick conscripts however showed only a minor change in adenovirus antibody titres during the same time

Fig 5 shows that no such serological conversion was observed with ECHO 28 neutralizing antibodies neither in the controls nor in the sick conscripts in spite of that this virus was also prevalent during this period These findings emphasize that a major outbreak of adenovirus infection can occur as silent infections The determinant for break through in disease is unknown and may either reside in virus properties or in environmental factors

Double Infection

Evidence for a double infection with two viruses was only secured from 8 cases in total In the autumn 1961 one of the Coxsackie A 21 infections was accompanied by an infection with Herpes simplex virus

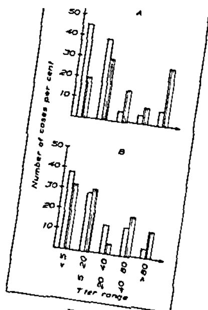


Fig 4

The distribution of adenovirus complement fixing titres in A controls and B sic[k] conscripts Unfilled staples record titres obtained in sera collected during the autum[n] 1962 Striped columns give the titre in sera from the winter 1963

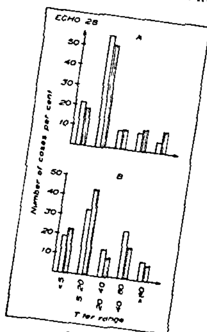


Fig 5

The distribution of ECHO 28 neutralizing antibody titres in A controls and B s[ick] conscripts Unfilled staples record titres obtained in sera collected during th[e] autumn 1962 Striped columns give the titres in sera from the winter 1963

and another case in the autumn 1962 had concurrent infections of adenovirus 4 and Herpes simplex virus. It appears likely that the Herpes simplex virus infections were secondary to the others. During the winter 1962 one case showed a simultaneous infection with para-influenza type 3 and Coxsackie A 21 and during the same period a concurrent infection with ECHO 28 and Adenovirus type 2 was observed. Finally during the epidemic outbreak of influenza type A 2 4 cases had serologic evidence of concurrent infection with parainfluenza type 1. It is of interest that the physician who examined the conscripts recognized two different types of disease during this outbreak.

Bacterial secondary infections must have been rare because in no case was evidence secured in favour of a bacterial secondary infection. On the other hand very early treatment with antibiotics was employed in a large number of cases.

Strain Variation of Viruses

The virulence of a virus for an animal host is dependent on many properties of the virus, the host and the environment (1). In order to elucidate the character of the influenza virus type A 2 strain responsible for the major outbreak in the winter 1963 the virus strain recovered was tested for reactivity against antibodies and horse serum inhibitors. The virus was isolated at the peak of the epidemic from one of the 60 cases admitted with typical influenza. As described above this outbreak was preceeded by a latent infection in the controls. Table 1 shows that the strain recovered has some characteristics of a + variant according to *Choppin & Tamm* (14) but also that an antigenic variation from the prototype strain exists. The antigenic relationship is R = 0.25 as defined by *Chu et al.* (15). Whether these findings have any bearing on the virulence of the strain is impossible to decide.

TABLE 1

Reactivity of the Influenza Virus Type A 2 Isolated from the Epidemic in 1963 to Anti Serum and Horse Serum Inhibitors

Serum	Virus: Infl A 2 prototype	Virus: Infl A 2 strain S488
Influenza A 2 prototype	160	320
Influenza A 2 strain S488 passage 9	10	320
Normal horse serum	<5	6400

Typing of Bacterial Strains

Since no type of hemolytic streptococci dominated during any of the investigation periods the results of typing have been compiled for the entire investigation period in Table 2. An untypable strain in group G tentatively referred to as type Uppsala was most common and recovered

during all investigation periods Several strains could be referred to group A and the types 10 and 22 were most common within this group Strains from the controls showed the same distribution as those from the sick conscripts As a rule hemolytic streptococci when present were isolated from the first swabs but in 10 cases they were only found at the second sampling In 16 cases the same type was isolated both from the first and the second swabs and different types were never recovered from the two samples

TABLE 2
The Distribution of Serological Types of Isolated Haemolytic Streptococci

β haemolytic streptococci group	Type	Number of cases 1st visit	Number of cases 2nd visit
A	1	1 (2)	
A	2	2 (3)	
A	3	1	1*
A	10	9 (10)	2 + 3*
A	12	3 (2)	1 + 1*
A	27		1
A	28	3 (1)	
A	22	8	1
A	untypable	2 (2)	2*
B		2	
C	21	2	2 + 1*
C	7	2	1*
F		2	
G	Uppsala	13 (13)	7*
G	NT	3	
G	16	1	
H		4	1
L		1	
N		1	
P		1	
Non typable		13 (13)	2 + 2*

* The first figure gives the number of strains only recovered at the second visit and the second figure denotes number of cases with the same type of β streptococcus on both occasions
( ) Denotes number of β streptococci isolated from healthy controls

Pneumococci were not common in this material Out of 31 isolated strains 20 could be typed and several types were represented In 12 cases the pneumococci were only isolated from the second swabs and in 15 cases recovered from both the first and the second samples *H influenzae* was not typed It is noteworthy that this organism was only isolated from the second swabs in 19 cases

As can be seen from Figs 2, 3 and Table 3 there was no marked concentration of bacteria at any particular time suggestive of an epidemic due to the organism in question the slight peak of β streptococci in November December 1960 might possibly suggest such an outbreak This was not confirmed, however, by the clinical findings

In some cases it was possible to isolate two or more species of bacteria

TABLE 3

Percentage Distribution of Potential Bacterial Pathogens in Sick Conscripts During the Entire Investigation Period 1960 1963

	Staph aureus %	β streptococci %	*H influenzae* %	Pneumococci %	Meningococci %
Autumn 1960					
Nose and throat	17.0	1.9	9.4	–	–
Throat	22.6	37.2	9.4	3.8	–
Nose	15.1	–	7.5	11.3	–
Negative	45.3	60.4	73.6	84.9	100
Total positive specimens	54.7	39.6	26.3	15.1	–
Winter 1961					
Nose and throat	7.6	–	3.0	1.5	–
Throat	25.8	22.7	4.5	3.0	–
Nose	15.2	–	7.6	9.1	–
Negative	51.5	77.3	84.8	86.4	100
Total positive specimens	48.6	22.7	15.1	13.6	–
Autumn 1961					
Nose and throat	18.1	–	–	–	–
Throat	22.7	13.6	4.5	–	4.5
Nose	9.1	–	4.5	4.5	–
Negative	50.0	86.4	90.9	95.5	95.5
Total positive specimens	49.9	13.6	9.0	4.5	4.5
Winter 1962					
Nose and throat	16.2	–	–	1.4	4.1
Throat	23.0	20.3	4.1	1.4	5.4
Nose	5.4	–	5.4	9.5	–
Negative	55.4	79.7	90.5	87.8	90.5
Total positive specimens	44.6	20.3	9.5	12.3	9.5

TABLE 3 (cont)

	Staph aureus %	β streptococci %	*H influenzae* %	Pneumococci %	Meningococci %
Autumn 1962					
Nose and throat	22 7 (15 3)	(2 0)	– (3 0)	– (1 0)	– (1 0)
Throat	18 2 (24 5)	18 2 (17 3)	(3 0)	– (2 0)	– (3 0)
Nose	18 2 (10 2)	– ( )	22 7 (7 1)	– (4 1)	– (2 0)
Negative	40 9 (50 0)	81 8 (80 6)	77 3 (86 7)	100 (92 9)	100 (93 9)
Total positive specimens	59 1 (50 0)	18 2 (19 4)	22 7 (13 3)	– (7 1)	– (6 1)
Winter 1963					
Nose and throat	16 1 (21 3)	(–)	3 2 ( )	(1 9)	– (–)
Throat	19 4 (32 4)	24 2 (20 4)	(1 9)	– (0 9)	– (–)
Nose	6 5 (12 0)	( )	6 5 (2 7)	4 8 (1 9)	1 6 (–)
Negative	58 1 (33 3)	75 8 (79 6)	90 3 (95 4)	95 2 (95 4)	98 4 (100)
Total positive specimens	41 9 (66 7)	24 2 (20 4)	9 7 (4 6)	4 8 (4 6)	1 6 (–)

( ) Denotes percentage recovery in controls at the same period

from the same individual the most common finding be[illegible]
in combination with some other organism

In conclusion it appears that the potentially path[illegible]
studied were not the primary cause of any of the dis[illegible]
except for single cases of infection with hemolytic strep[illegible]

Effect of Antibacterial Therapy

Penicillin treatment was employed in a large number of cas[illegible]
the treatment was usually started early in the infection Th[illegible]
treatment are shown in Table 4 For purposes of comparison [illegible]
subjects with potential pathogens in the respiratory tract are [illegible]
The effect of penicillin upon penicillin sensitive organisms was ad[illegible]
quate This was particularly true of pneumococci and m[illegible]
which were almost completely eliminated The treatment was [illegible]
less efficient with hemolytic streptococci since 7 of 30 treat[illegible]
were still carrying this organism when the second sample [illegible]
Staph aureus was eliminated in some cases but not so eff[illegible]
ol served previously (3) Therapy increases recovery of *H* [illegible]
pointed out earlier (4) This organism was isolated in the [illegible]
men in 7 out of 13 treated cases and in another 13 treated [illegible]
fluen ae was recovered only in the second specimen It is dif[illegible]
cult to establish whether bacteria isolated in the second [illegible]
swabs at an interval of 5 days might be an expression of [illegible]
or an increased yield expected after duplicate samp[illegible]
factors were probably at play judged from antibody est[illegible]
not however be proved that the acquired *H influen* [illegible]
clinical importance

TABLE 4

The Effect of Ant bacter al Therapy on the Isolat on of Ba[illegible]
S ck Conser pts

T pe of bacter a	Only at 1st visit		Both at 1st and 2nd v[illegible]	
	Treated	Not treated	Treated	Not treated
N men ng	3	3	0	1
Pneumococci	13	8	0	3
Hem l ti strept cocci	30	13	7	9
H nfl ien ae	13	11	7	9
Staph aureus	34	21	26	4

Correlation between Clinical Syndrome and Microbial [illegible]

It was already pointed out in previous communica[illegible]
correlation exists between clinical syndrome and et[illegible]
study clearly demonstrates that none of the pathog[illegible]
clinical picture Fig 6 shows the distribution of clin[illegible]

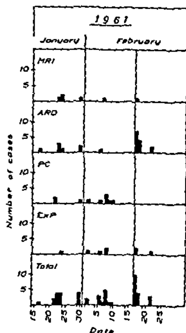


Fig 6
Distribution of different clinical syndromes in the epidemic of adenovirus type 4 infections in the winter 1961

defined in Methods in the adenovirus epidemic during the winter 1961 All the four syndromes are represented and although conjunctivitis was observed at the start of the epidemic the clinical symptomatology was different at the end of the epidemic In general, however, acute respiratory disease (ARD) appears to be more frequently observed when adenovirus and influenza virus are associated with the disease Minor respiratory illness was found to account for the major fraction of the diseases, when ECHO 28 and Coxsackie A 21 was isolated Finally exsudative pharyngitis appears to be associated with streptococcal disease as seen during the autumn 1960 period in Fig 2

DISCUSSION

The controlled study during the autumn of 1962 and the winter of 1963 demonstrated that only in the major outbreak of influenza A the virus could be statistically bound to the disease from an etiological point of view It is, however, always difficult to get control groups which cover an outbreak to complete satisfaction The first case of this outbreak of influenza A occurred late in January and two controls showed serological conversion among the 29 cases who gave their first bloodspecimen on the 12th of February The 19th and 26th of February the first bloodspecimen was taken in 55 new controls and 15 of them showed serological conversion but the convalescent serum was drawn as late as

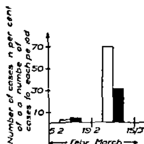


Fig 7

The incidence of influenza virus type A 2 infections in controls (filled columns) and sick conscripts (unfilled columns) during the investigation period February March 1963

the 11th and 12th of March The epidemic culminated four to five days earlier and two of the controls fell ill on the 7th of March and were consequently transferred to the list of sick conscripts It is evident that the 15 infected controls could have been infected as late as the first week of March The control groups in Fig 3 are in time referred to the date of the first serum specimen taken but Fig 7 with somewhat changed time intervals may give a more true picture of the outbreak and the relationship between the number of cases and infected controls It appears however that the infectious agent was disseminated in the population as a whole and only after some passages caused a more extensive outbreak which might have been caused by a change in virulence of the virus Another reason might have been that the out break was preceded by a time of accumulation of virus within the population probably increasing the size of the infectious doses spread

Thus the criteria for the acceptance of an infectious agent as the cause of a disease in the definitions given earlier in the paper might be valid only for agents which do not cause silent infections

If so only several years observations of a number of outbreaks in which a certain pathogen is proven to be closely associated with a disease will give reasonable evidence of the etiological significance of this agent

The outbreaks described in this series in which adenoviruses Coxsackie virus type A 21 and ECHO virus type 28 were involved should therefore be considered as contributions to the already existing observations which may accumulate to the conclusion that these pathogens are of etiological significance

The major fraction of virus infections in the military population described in this series was caused by epidemic outbreaks of a single virus which only reappears in the population after a variable length of time This is observed in spite of the fact that the conscripts were only stationed at the camp for 9 12 months and the camp was reoccupied by a new force after this period The different epidemics of virus infections observed have been recorded in Fig 8 for the entire 4½ years

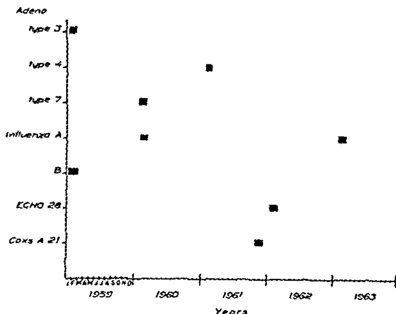


Fig 8

The major epidemics of virus infection during the entire investigation period 1959–1963

period 1959–1963 of this study In the winter 1959 a small outbreak of adenovirus type 3 occurred concurrently with an outbreak of influenza type B Neither of these viruses reappeared again During the winter 1960 an epidemic of adenovirus type 7 occurred concurrently with an outbreak of influenza virus type A 2 The latter virus reappeared after 3 years in an epidemic in the winter 1963 but adenovirus type 7 did not reappear In the winter 1961 an extensive epidemic of adenovirus type 4 was observed and only single cases of sick conscripts infected with this virus was found thereafter On the other hand the controls in the period 1962–1963 must have been latently infected with an adenovirus Thus, in one case the same virus is associated with overt illness in the other period it runs a silent course Single outbreaks were also observed of ECHO 28 and Coxsackie A 21 infections This reappearance of viruses in the military population makes it difficult to select the proper virus species for a prospective vaccine program

Of bacteria isolated from the respiratory tract during the six periods of investigation, only those which were isolated from swabs taken on the first examination before the conscripts had been in contact with the hospital environment were recorded The cultures from the second set of swabs are affected by treatment and possibly also by nosocomial infection

No particular organism predominated in the swabs but *Staph aureus* was most commonly present The incidence of other potential pathogens varied at the different investigation periods The highest frequency of

hemolytic streptococci 39 6 per cent was recovered during the autumn 1960 and the lowest, 13 6 per cent during the autumn 1961 *Haemophilus influenzae* was most common during the autumn 1960 with 26 3 per cent and the autumn 1962 with 22 7 per cent The incidence was low— around 9 per cent—during the other periods The frequency of pneumococcal isolates was unexpectedly low During the autumn 1962 no pneumococci were isolated but during the autumn 1960 15 1 per cent of the conscripts carried this organism It may also be noted that meningococci were isolated during three periods at a frequency of 4 5 per cent, 9 5 per cent and 1 6 per cent respectively No cases of meningitis were observed During the autumn 1962 and the winter 1963 controls were included in this study The frequency of potential pathogens among this group was about the same as in the group of sick conscripts No significant difference in isolation rates between the groups was observed During the autumn 1962 pneumococci and meningococci were isolated in 6 1 and 7 1 per cent respectively of the controls but none of the diseased carried these organisms Bacteria thus appears to cause only a negligible fraction of the respiratory diseases

SUMMARY

The studies on the acute respiratory infections in military personnel which stated in 1959, have been concluded with an evaluation of the results of a study during the years 1960–1963 Four outbreaks of epidemiological interest were observed associated with adenovirus type 4 in the winter 1961, Coxsackie virus type A 21 in the autumn 1961, ECHO virus type 28 in the winter 1962 and influenza virus A 2 in the winter 1963

The criteria for the acceptance of a pathogen as the cause of disease was discussed It has been stated that the introduction of control groups in a study might not fully elucidate this question After some reservations on the etiological significance of the findings it was found that the causative organisms were tentatively identified in about 43 per cent of the cases Bacteria seems to cause only a negligible fraction of the acute respiratory diseases

REFERENCES

1 Conference on newer respiratory disease viruses Am Rev of Resp Dis *88* 1 1963
2 *Dinter Z Ekelund H Laurell G Lindbom G Lofstrom G Philipson L & Wesslén T* Etiology of respiratory tract infections in military personnel Virological findings Acta path et microbiol scandinav *53* 375, 1961
3 *Dinter Z Ekelund H Laurell G Lindbom G., Lofstrom G Philipson L. & Wesslen T* Etiology of respiratory tract infections in military personnel Bacteriological findings Acta path et microbiol scandinav *53* 383 1961
4 *Dinter Z Ekelund H Laurell G Lindbom G Löfstrom G Philipson L & Wesslen T* Etiology of respiratory tract infections in military personnel 3 Correlation between clinical findings and association of virus and bacteria in infection *Acta path et microbiol scandinav* *54* 449 1962

5 *Kjersgaard R Lindbom G Dinter Z & Philipson I* Etiology of respiratory tract infections in military personnel 4 The recovery of Coxsackie A 21 virus from cases with minor respiratory disease Acta path et microbiol scandinav 56 465 1962

6 *Kjersgaard R Lindbom G Dinter Z & Philipson L* Etiology of respiratory tract infections in military personnel 5 The recovery of ECHO 28 from cases with minor respiratory illness Acta path et microbiol scandinav 59 537 1963

7 *Eagle H* Amino acid metabolism in mammalian cell cultures Science 130 452 1959

8 *Youngner J S* Monolayer tissue cultures I Preparation and standardization of suspensions of trypsin dispersed monkey kidney cells Proc Soc Exp Biol and Med 85 202 1954

9 *Bodian D* Simplified method of dispersion of monkey kidney cells with trypsin Virology 2 575 1956

10 *Vogel J & Shelokov A* Adsorption haemagglutination test for influenza virus in monkey kidney tissue culture Science 126 358 1957

11 *Chanock R M Parrot R H Cook M K Andrews B E Bell J A Reichelderfer T Kapikian A Z Mastrota F M & Huebner R J* Newly recognized myxoviruses from children with respiratory disease New Engl J Med 258 207 1958

12 *Mogabgab W J* 2060 virus (ECHO 28) in KB cell cultures Characteristics complement fixation and antigenic relationships to some other respiro viruses Amer J Hyg 76 15 1962

13 *Crieble H C Jackson C G Dowling H F Sekela D H & Anderson T O* The etiology of common respiratory infections in a civilian adult population Am J Med Sci 235 245 1958

14 *Choppin P W & Tamm I* Studies of two kinds of virus particles which comprise influenza A 2 virus strains I Characterization of stable homogenous substrains in reactions with specific antibody mucoprotein inhibitor and erythrocytes J Exp Med 112 895 1960

5 *Chu C M Andrews C H & Gledhill A W* Influenza in 1948 1949 Bull World Health Org 3 187 1950

Acta path et microbiol scandinav 66 227-238, 1966

The Municipal Virological Laboratory, Gothenburg Sweden

PROPERDIN AND THE ANTIBODY-EFFECT ON *TOXOPLASMA GONDII*

By

ÖRJAN STRANNEGÅRD and ERIK LYCKE

Received 31 viii 65

In a previous study (10) a cell culture method was used to describe the effect on the infectivity of toxoplasma by specific antibodies and activator factors (properdin, complement and Mg^{++}) The results indicated that properdin was an important cofactor for obtaining an effect of antibodies and that, in addition, it might have an effect *per se* on the toxoplasma parasites Previous investigations by others (1, 4, 5), using the Sabin-Feldman dye test for studies on the effect exerted on toxoplasma by properdin, have given somewhat conflicting results

The cell culture method described by *Lycke & Lund* (8, 9) is in some respect more sensitive than the dye test for a demonstration of anti-toxoplasma activity The dye test reveals morphological and chemical alterations of the parasites With the cell culture method anti-toxoplasma activity is demonstrated by the effect on the capacity of the parasites to penetrate the host cells The present report describes further studies on the rôle of properdin for the antibody effect on Toxoplasma gondii The effect on the capacity of penetration and the appearance of morphological alterations are used as parametres

MATERIAL AND METHODS

Parasites The RH strain of *Toxoplasma gondii* was used The preparation of parasite suspensions for the cell culture tests was the same as described previously (8) If not otherwise stated parasite suspensions containing 5 × 10⁶ to 1 × 10 parasites per ml were used in the experiments

The cultures Standardized cultures of HeLa cells in Gey chambers were used (8) During the outgrowth phase the cells were cultured in Hanks solution containing antibiotics 0 5 per cent lactalbumin and 20 per cent heat inactivated human activator serum

The immune serum One batch of human immune serum was used throughout the experiments It was obtained from a 24 year-old man who had been affected by the lymphoglandular form of toxoplasmosis for at least 3 months This serum had a dye test titre of 8000 and a titre in the complement fixation test of 256 The serum was heat treated at 56° C for 30 min before use In some experiments the 7S fraction of the serum was used instead of whole serum This fraction was ob

This study was supported by a *PHS research grant AI 05074-03 from the National Institute of Allergy and Infectious Diseases Public Health Service USA* The skillful technical assistance of Miss Mona Eriksson is gratefully acknowledged

tained by gel filtration on a Sephadex (R) G 200 column according to the method described by *Flodin & Killander* (3) The 19S part of this fractionation contained toxoplasma antibody activity as measured in the dye test in a concentration of about one hundredth of that found in the 7S fraction

The amount of antibody which gave morphological alterations (MA) when used together with activator serum in 50 per cent of added toxoplasma parasites is, in the following referred to as one antibody unit The technique used for determination of MA is described below

The activator sera Fresh human sera from healthy donors were used These sera were negative in complement fixation tests and in dye tests when tested untreated and undiluted i e the percentage of parasites showing MA did not exceed 80 They enabled a positive reference serum to react with a constant dye test titre in repeated tests The sera were stored at —70° C before use

The properdin The properdin reparation (RdTI) employed was supplied by AB KABI Stockholm, Sweden The preparatory technique which had been used was principally, the one described by *Spicer et al* (17) From a serum pool of retroplacental serum Cohn s fraction I was prepared This fraction was subsequently precipitated by tetrametaphosphate and finally lyophilized The preparation contained 25 3 per cent protein amounting to 0 3 per cent of the total protein content in the original serum pool The amount of properdin in the lyophilized preparation was determined to 16 000 units per gram [1]

In starch gel electrophoresis the preparation was found to have a mobility corresponding to that of human gammaglobulin Anodal as well as cathodal components were demonstrable

The properdin preparation was tested for contents of complement factors using R1 R2 R3 and R4 sera prepared according to *Kabat & Mayer* (6) The preparation tested in final concentrations of up to 200 units per ml was not found to contain any C 1 C 2 C 3 or C 4 factors At high concentrations of the preparation an anti complementary effect was observed One hundred units of properdin were required for the inhibition of one CH_{50}

For determination of the possible presence of toxoplasma immune antibodies in the properdin the preparation was tested in high concentrations in complement fixation tests using toxoplasma antigen made from chorioallantoic membranes of infected chick embryos No evidence of an occurrence of toxoplasma antibodies was obtained in the direct test nor in the indirect test described by *Rice* (15)

Test on morphological alterations (MA) of toxoplasma parasites MA of toxoplasma parasites were studied by means of phase contrast microscopy and were readily seen without any staining procedure Parasites with MA appeared as slender granular dark crescents with a clearly visible nucleus Parasites were considered unaffected if they were homogenously bright and of a more bean like appearance In each test determinations of the percentage of parasites showing MA were based on observations of 200 parasites or more

The tests on MA were performed with one part of antibody dilution 2 parts of activator serum and one part of parasite suspension containing 5×10^6 to 1×10^7 parasites Readings were performed after incubation at 37° C for one hour When properdin was included in the tests it was added as dry substance

The cell culture test The test reagents were mixed with an equal volume of toxoplasma parasites incubated for one hour at 37° C and then inoculated into HeLa cell cultures as described previously (8) After incubation of the cultures for 19 hours at 37° C the relation between the number of parasites having penetrated the host cells and the number of exposed host cells was determined The figures expressing this relationship *is referred to as the relative number of infectious units* (RNIU)

Absorption with zymosan The zymosan absorptions were performed either at 17° or at 37° C or at both temperatures as described by *Wardlaw & Pillemer* (19) The zymosan preparation used was obtained from Mann Research Laboratories Inc, New York 6 N Y

---

1 The assay of properdin was kindly performed by Dr *Anna Britta Laurell* Institute of Bacteriology Lund Sweden

RESULTS

The Effect of Properdin on Toxoplasma in the Absence of other Serum Components

Toxoplasma parasites were exposed to properdin for one hour at 37° C Concentrations ranging from 1 6 to 160 units/ml of properdin were used The percentage of parasites showing MA and the RMIU was determined

Properdin did not cause any morphological alterations of the parasites No lysis of parasites was observed, and no reduction in the number of infective, *i e* penetrating, parasites was found

In one experiment, the multiplication rate of toxoplasma in the presence of properdin was followed by means of readings after 6, 10, 14 and 19 hours of incubation as described previously (9) Properdin in a concentration of 100 units per ml did not have any effect on the multiplication rate

The Antibody Activating Effect of Properdin in the Presence or Absence of other Activator Components

In the absence of activator serum, the heat treated reference human antiserum regularly caused MA in 10 to 30 per cent of the exposed parasites when it was used, also in high dilutions, together with high concentrations of properdin To see whether this effect was dependent upon remaining minute amounts of complement in the serum, experiment were performed using the 7 S fraction instead of whole serum The results obtained are listed in Table 1 This table also includes results obtained when activator serum was added to the properdin-antibody mixtures

TABLE 1

The Effect of Various Concentrations of Properdin Used together with Toxoplasma 7 S Antibodies

Units of properdin added	0	10	50	100	150	200
% parasites with MA in absence of activator	3	3	3	14	12	5
% parasites with MA in presence of activator	91	92	83	76	44	26

Four 7 S antibody units properdin in various concentrations and toxoplasma parasites were mixed in one series together with activator serum and in another without addition of activator After one hour's incubation at 37° C the percentage of parasites with morphological alterations (MA) was determined

As illustrated by the number of parasites showing MA, the properdin preparation in high concentrations had a certain antibody-activating effect also *in the absence of the other activator components* *With* concentrations of properdin exceeding 100 units per ml this reaction

however, was inhibited Inhibition was also demonstrable when other activator components were present and was found to be independent of the concentration of the activator serum used The degree of inhibition demonstrable at a final activator serum dilution of 1 8 was the same as that observed using undiluted activator (i e when dilutions of parasites and antiserum were performed with activator serum) Properdin was inhibitory also when it had no detectable anticomplementary activity as shown by a complement titration

TABLE 2

The Antibody Activating Effect of Properdin Comparison of Results Obtained by Cell Culture Tests and Tests for Demonstration of Parasites with Morphological Alterations

Sample	RNIU	Parasites with MA per cent
Activator	45 1	23
Antibodies	43 6	12
Antibodies + activator	34 9	96
Antibodies + activator + properdin 50 units	33 7	90
Antibodies + activator + properdin 150 units	29 4	77
Antibodies + properdin 50 units	41 6	19
Antibodies + properdin, 150 units	40 8	8
Properdin 150 units	50 0	4
Hanks' solution + lactalbumin	49 6	4

The figures expressing the number of properdin units refer to final concentrations per ml Four 7 S antibody units were used Toxoplasma parasites were added to the samples indicated After one hour's incubation at 37° C, the percentage of parasites with morphological alterations (MA) was determined In addition cell cultures were inoculated and the relative number of infective units (RNIU) was assayed

Similarly, in the cell culture tests properdin regularly evoked a slight antibody-activating effect, also in the absence of activator serum (Table 2) The cell culture tests did not demonstrate any inhibition of the antibody-activating effect of properdin using concentrations of properdin which were inhibitory according to results obtained by obs vations of parasites with MA

The Antibody-Activating Effect of Various Properdin Concentration

Two antibody units were mixed with an activator serum absorbed with zymosan at 17° C for one hour and with properdin in concentrations ranging from 1 to 250 units/ml After incubation of the mixtures with parasites at 37° C for one hour, the percentage of parasites showing MA was determined The results are illustrated in Fig 1 The optimal concentrations of properdin were found to be in the range of 5 to 50 units per ml At higher concentrations of properdin the reaction leading to MA of the parasites was inhibited The lower percentage of morphologically altered parasites found with concentrations of pro

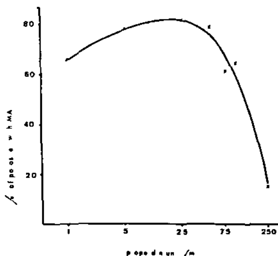


Fig 1

Antibody activating effect at various concentrations of properdin
Two antibody units were mixed with an activator serum absorbed with zymosan and with properdin in various concentrations The mixtures were incubated with toxoplasma parasites for one hour at 37° C at the percentage of parasites showing morphological alterations (MA) was determined

perdin exceeding 50 units per ml could not be attributed to an increased lysis of damaged parasites as no reduction in the absolute number of parasites was observed

The Effect of Properdin at Various Antibody Concentrations

Three series of mixtures of the reference antitoxoplasma serum activator serum and properdin were made All mixtures contained the same concentration of activator serum and all the three series contained antiserum in dilutions ranging from 1/4 to 1/32768 (4096—0 5 units of antibody) To the mixtures in two of the series properdin was added in a concentration of 15 and 150 units per ml respectively The third series received no properdin Each mixture was tested for ability to produce MA in exposed parasites The results are illustrated in Fig 2 The inhibitory effect of 150 units of properdin per ml was demonstrable irrespective of the antibody concentration used

The Effect on Toxoplasma of Activator Serum Absorbed with Parasites and or Zymosan

An activator serum was used which caused MA in 43 per cent of exposed parasites when employed undiluted and without previous heat treatment A portion of the serum was absorbed with parasites 3 times at 0° C for 45 min Each time washed toxoplasma parasites were added to the serum to a final concentration of 10 parasites per ml The parasites were removed by centrifugation at 1 000 g for 10 min Another

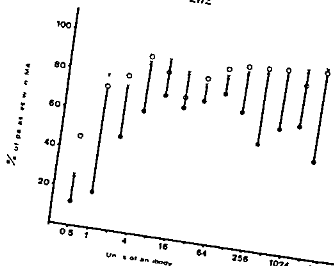


Fig 2

The influence of the concentration of specific antibody on the antibody activator effect of properdin

Various concentrations of toxoplasma antibodies were mixed with an activator serum Properdin (15 units open circles 150 units filled circles) or veronal buffer (crosses) were added to the mixtures After incubation with toxoplasma parasites at 37° C for one hour the percentage of parasites showing morphological alterations (MA) was determined

portion of the serum was absorbed with zymosan at 17° C (1 hour) and at 37° C (½ hour) according to the procedure used to obtain RPb (19) Finally a third portion was absorbed with parasites as well as with zymosan The results of the absorptions and the effect of adding to absorbed serum a small amount of antibody, which, pretested together with activator, caused no MA in the concentration used are shown in Table 3

TABLE 3

The Effect of Absorption of Activator Serum with Toxoplasma Parasites and/or Zymosan

Absorption	Additive	RNIU	MA
None	None	35 2	43
Parasites	None	39 6	39
Zymosan	None	59 8	7
Parasites + Zymosan	None	58 3	5
Parasites	Antibodies	15 1	35
Zymosan	Antibodies	61 4	6

A human serum was absorbed three times with parasites at 0° C using 10 million parasites per ml Absorptions with zymosan were performed at 17° C and at 37° C (19) One sample was absorbed with parasites as well as with zymosan To two of the samples a minute amount of immune antibody (1/4 unit) was added before tests Toxoplasma parasites were added to the samples and after one hour's incubation at 37° C the percentage of parasites with morphological alterations (MA) was determined In addition cell cultures were inoculated and the relative number of infective units (RNIU) was assayed

Absorption with zymosan was effective in reducing the antitoxoplasma activity of the serum whereas absorption with toxoplasma parasites was much less potent Absorption with both zymosan and parasites was no more effective than the absorption with zymosan alone Addition of the small amount of antibody (1/4 unit) to the parasite absorbed serum did not significantly change the percentage of parasites showing MA, but reduced markedly the number of infective parasites, demonstrating the difference in sensitivity between the two tests On the other hand, if the serum was previously absorbed with zymosan, instead of with parasites, the addition of small amounts of antibody gave no effect either in the cell culture tests The results indicate the mutual dependence of antibodies and factors removable from serum with the zymosan for obtaining antitoxoplasma activity

The Effect of Adding an Excess of Properdin at Various Times Following the Addition of Antibodies and Activator to Toxoplasma

Two antibody units were mixed with activator serum and toxoplasma parasites At various times samples were taken from the mixture and properdin was added to the samples to give a final concentration of 160 units of properdin per ml Immediately before adding the properdin, and one hour later, the percentage of parasites showing MA was determined The results are listed in Table 4

TABLF 4

The Effect of Adding Fxcess Properdin at Various Instances after mixing Toxoplasma with Antibodies and Activator

Time in minutes	Parasites with MA per cent	
	Before the addition of properdin	One hour after the addition of properdin
1	3	62
10	70	78
30	79	90
60	98	99

Two antibody units activator and toxoplasma parasites were mixed Samples were drawn from the [illegible]

As found in experiments described above, an excess of properdin was found to have an inhibitory effect This was most evident if the excess of properdin was added to the parasites simultaneously with the antibody-activator mixture, but a similar effect could be demonstrated even when properdin was added as late as 30 min after the mixing of parasites with antibody and activator No reversal to normal morphology of parasites showing morphological alterations was observed

The Effect of Properdin on the Attachment of Antibodies to Toxoplasma

In one series of tests, two antibody units were mixed with various concentrations of properdin and in another series antibodies and properdin were, in addition mixed with activator serum After incubation of the mixtures with toxoplasma parasites at 37° C for one hour the percentage of parasites showing MA was determined The parasites were then removed by centrifugation at 440 × g for 20 min and the supernatants obtained were heat treated at 56° C for 30 min Activator serum was added to the supernatants which were subsequently tested for activity causing MA of toxoplasma parasites

TABLE 5

The Effect of Excess Properdin on the Attachment of Specific Antibodies to Toxoplasma Parasites

Sample	Properdin units/ml	Parasites with MA per cent	Antitoxoplasma activity in supernatant
Antibodies + activator	None	91	55
Antibodies + activator	10	93	58
Antibodies + activator	100	95	69
Antibodies + activator	250	50	71
Antibodies	None	19	46
Antibodies	10	27	35
Antibodies	100	21	47
Antibodies	250	16	59

Properdin in various concentrations was added to the samples indicated in the table Two antibody units were used in each sample The samples were all ne l to react for one hour at 37° C with toxoplasma parasites whereupon the percentage of parasites with morphological alterations (MA) was determined The parasites were removed by centrifugation the supernatants were heated at 56° C for 30 min and tested for content of antibody by adding parasites and activator The results of these tests indicating antitoxoplasma activity in the supernatants refer to percentage of parasites with MA

As shown in Table 5 high concentrations of properdin inhibited the action of the antibodies whether or not activator serum was present The antitoxoplasma activity of the supernatants increased in relation to the amount of properdin used Analogous results were obtained if the properdin was added to parasites washed in veronal buffer after previous treatment at 37° C for 20 min with antibodies or with antibodies and activator

The Effect of Antibody on Toxoplasma Parasites Previously Treated with Properdin or with a Mixture of Properdin and Activator

Toxoplasma parasites were added to two series of test tubes both series containing properdin in various concentrations and one of the series in addition activator serum After incubation at 37 C for 30

min the parasites were sedimented by centrifugation at 440 × g for 10 min The supernatants were discarded, and each sediment was resuspended in veronal buffer containing 16 antibody units After a subsequent incubation for 30 min at 37° C the percentage of parasites showing MA was determined

TABLE 6

The Effect of Treatment of Toxoplasma Parasites with Properdin or with Properdin and Activator before Addition of Antibodies

Sample	Parasites with MA per cent
Properdin 10 units/ml	6
Properdin, 50 units/ml	10
Properdin 200 units/ml	39
Activator + properdin 10 units/ml	8
Activator + properdin, 50 units/ml	45
Activator + properdin, 200 units/ml	62
Activator	7
Veronal buffer	5

To the samples indicated toxoplasma parasites were added After 30 min at 37° C the parasites were sedimented at 440 g for 10 min the supernatants were discarded and the parasites were resuspended in veronal buffer containing 16 antibody units per ml After additional 30 min at 37° C the percentage of parasites with morphological alterations (MA) was determined

Table 6 shows that under the experimental conditions mentioned no inhibitory effect of high concentrations of properdin was observed After exposure of the parasites to increasing concentrations of properdin the percentage of parasites exhibiting MA was correspondingly increased Thus properdin seemed to sensitize the parasites, making them more vulnerable to the subsequent action of antibody It should be noted that incomplete removal of properdin-containing supernatant from the sedimented parasites would not account for the results obtained Thus results were not different if the parasites were washed in veronal buffer before the addition of antibodies

DISCUSSION

The concept of the properdin system has been a subject of much debate *Pillemer et al* (14) claimed that properdin was to be regarded as a serum factor with a multitude of non-specific biological activities Properdin, which *required complement and magnesium for its action*, was considered to be distinct from antibody The concept was initially challenged by *Nelson* (12) who claimed that the activities ascribed to the properdin system could be fully explained as functions of classical antibody in combination with complement factors Properdin was considered to be antibody capable of reacting with zymosan and thus the apparent lack of specificity was explained as being due to cross-reactivity with a multitude of antigens *Wedgwood* (20) has suggested that

properdin . . . . ad functioning in a . . . the action of prop . . . . antigen with specific antibody

Gronroos (4) has shown that properdin is an integral part of the so-called activator system, necessary for the action of toxoplasma antibodies *Feldman* (1) confirmed these findings and showed that properdin *per se* had no anti-toxoplasma effect Previously, *Roth* (16) had found the activator system contained at least 3 complement factors, C'2, C'3 and C'4 According to *Feldman* (2) the activator system is identical with complement + properdin + magnesium ions, *i e* the properdin system

Recent investigations by *Metzger et al* (11) suggest that the properdin system is of vital importance for the inhibition *in vivo* of the toxoplasma infection Administration of zymosan to experimentally infected animals was found to result in a prolonged parasitaemia and in a considerably decreased ability to resist toxoplasma infection

In a previous paper (10), we suggested that properdin itself might have an antitoxoplasma effect The results of the present investigation do not support this assumption The action of properdin is apparently dependent upon the interaction of the toxoplasma parasites with specific antibodies The antibody concentration necessary for this interaction may, however, be extremely small, being non-detectable in the dye test The finding of an antibody requirement is consistent with the results obtained by *Toussaint & Muschel* (18) who found that antibody was necessary for the neutralization of coli T bacteriophages by "normal" human serum Similarly, *Osawa & Muschel* (13) found that antibody was required for the bactericidal action of "normal" serum

The evident antitoxoplasma effect evoked by the combined action of properdin and antibody in the absence of demonstrable complement indicates a mode of action of properdin different from that in a bactericidal system It cannot be excluded, however, that the preparations of properdin and antibodies contained small amounts of haemolytic complement not detectable by the methods used It has previously (10) been found that, although there was a reduction in the activating capacity when complement components were removed, the activation of antibody seemed not absolutely to require the presence of all the components

The antitoxoplasma reaction was dependent upon the concentration of properdin and in high concentrations properdin was found inhibitory to the reaction The inhibition phenomenon has previously been observed by *Feldman* (1) We found that the inhibition occurred independently of the concentration of antibody used This observation and the finding that the inhibition could also be established when properdin was added to the parasites later than the antibodies, indicate that properdin and immune antibodies are different entities

Inhibition of the antibody activating effect in the presence of high concentrations of properdin would be obtained if properdin prevented the antibodies from reacting with the toxoplasma antigens Similarly, inhibition would occur if an additional component, e g a complement factor necessary for the antibody activation was made unavailable to the antibodies by reaction with excess properdin As a third possibility, the inhibition might be explained as the result of a dissociation of antibodies at high concentrations of properdin from the reactive sites of the toxoplasma parasites Even a 8000 fold increase in the concentration of immune antibodies did not overcome the inhibition This is incompatible with the possibility that the inhibition was caused by a binding of antibody to properdin The properdin preparation used was found to have a certain anticomplementary activity when it was used in high concentrations However, the inhibition also occurred when the amount of added activator serum was increased Thus it is improbable that the inhibition of antibody activation were due to an anticomplementary effect of the properdin preparation

A more plausible explanation of the inhibition would be a dissociation of antibodies from the toxoplasma antigens in the presence of a high concentration of properdin This assumption is supported by the observation of an increased antitoxoplasma activity in the parasite suspending medium after treatment with properdin of antibody coated parasites It has previously (10) been shown in cell culture tests that damage to the parasites reflected in their reduced ability to penetrate the host cells can be demonstrated before morphological alterations of parasites are detectable On the assumption that the penetrating capacity of the parasites is irreversibly damaged before any morphological alterations appear the failure to demonstrate inhibition of the antibody effect on toxoplasma by the cell culture tests may be used in further support of the dissociation hypothesis The assumption that properdin dissociates the antibodies from the toxoplasma antigens corresponds well to the results obtained by *Ludvik et al* (7) who found that ferritin labelled antibodies attached to toxoplasma parasites disappeared after the addition of activator serum

The results of pretreatment of parasites with properdin indicated that properdin reacted with the parasites which became more vulnerable to the subsequent action of antibody Properdin seems to be bound to the parasites without damaging the cell wall as properdin *per se* neither influences the parasite multiplication nor the penetration into its host cells

SUMMARY

The role of properdin for the antibody effect on *Toxoplasma gondii* was studied by means of microscopic observations of parasite morphology and by a cell culture test Properdin was found to combine with toxoplasma parasites sensitizing the parasites to the action of antibody

An antitoxoplasma activity in the absence of any demonstrable complement was observed when properdin was used together with antibody In addition to its antibody activating effect, properdin seemed to be able to dissociate antibodies attached to reactive sites of the toxoplasma parasites Thus properdin in high concentrations was inhibitory to the antibody effect on toxoplasma The inhibition occurred independently of the antibody concentration used and could also be established when properdin was added to the parasites later than the antibodies were added These results indicate that properdin and immune antibodies are different entities

REFERENCES

1 *Feldman H A* The relationship of toxoplasma antibody activator to the serum properdin system Ann N Y Acad Sci 66 263 267 1956
2 *Feldman H A* Studies on the accessory factor Discussion Human toxoplasmosis 219 Ed J Ch Siim Munksgaard Copenhagen 1960
3 *Flodin P & Killander J* Fractionation of human serum proteins by gel filtration Biochim biophys Acta 63 403–410 1962
4 *Grönroos P* *The action of properdin on Toxoplasma gondii* Ann Med Exp Biol Fenniae 33 310-315 1955
5 *Grönroos P* Discussion of the paper of Eichenwald H The laboratory diagnosis of toxoplasmosis Ann N Y Acad Sci 64 213 214 1956
6 *Kabat E A & Mayer M M* Exp Immunochemistry Charles C. Thomas Springfield Illinois chapter 4 159 165 second edition 1964
7 *Ludvik J Siim J Chr & Birch Andersen A* The ultrastructure of *Toxoplasma gondii after treatment with specific antibodies Communication* at the 7th Int Congr on Trop Med and Malaria Rio de Janeiro 1963
8 *Lycke E & Lund Ebba* A tissue culture method for titration of infectivity and determination of growth rate of *Toxoplasma gondii* 1 Acta path et microbiol scandinav 60 209 220 1964
9 *Lycke E & Lund Ebba* A tissue culture method for titration of infectivity and determination of growth rate of *Toxoplasma Gondii* 2 Acta path et *microbiol* scandinav *60* 221 233 *1964*
10 *Lycke E Lund E Strannegård O & Falsen E* The effect of immune serum and activator on the infectivity of *Toxoplasma gondii* for cell culture Acta path et microbiol scandinav 65 206–220 1965
11 *Metzger M Pr erwa Tetmajer A & Wojciechowski Z* The influence of zymozan cortisone and X rays on the course of experimental toxoplasmosis in white rats Arch Immunol Ther Exp *11* 227 233 1963
12 *Nelson R A Jr* *An alternative mechanism for the properdin system* J Exp Med *108* 515 535 1958
13 *Osawa E & Muschel L H* The bactericidal action of normal serum and the properdin system J Immunol 84 203 212 1960
14 *Pillemer L Blum L Lepow I H Ross O A Todd E W & Wardlaw A C* The properdin system and immunity I Demonstration and isolation of a new protein and its role in immune phenomena Science *120* 279 285 *1954*
15 *Rice C E* Some factors influencing selection of complement fixation method Parallel use of direct and indirect technics J Immunol 60 11 16 *1948*
16 *Roth W* Zur Wirkungsweise des Aktivatorserums auf *Toxoplasma gondii* Schweiz Z Allg Pathol *16* 914 918 1953
17 *Spicer D S Priester I I Smith F E C & Sanders B E* Properdin Preparation from plasma fraction I of the Cohn method J Biol Chem 234 838 840 1959
18 *Toussaint A J & Muschel L H* Neutralization of Bacteriophage by normal serum Nature *183* 1825 1827 1959
19 *Wardlaw A C & Pillemer L* The properdin system and immunity V The bactericidal activity of the properdin system J Exp Med *103* 553 575 1956
20 *Wedgwood R J* Properdin Immunological methods 25 41 ed J F Ackroyd Blackwell Scientific publications 1964

Acta path et microbiol scandinav 66 239–256 1966

The National Bacteriological Laboratory, Stockholm Sweden

THE IMMUNE RESPONSE TO VACCINATION WITH INACTIVATED POLIOVIRUS VACCINE IN SWEDEN

By

M BOTTIGER, L ARRO, H LUNDBECK and C-R SALENSTEDT

Received 18 ix 65

General vaccination with inactivated poliovirus vaccines has been performed in Sweden since 1957, vaccinations with live vaccine have been carried out only in small scale trials During the years 1957 to 1962 almost 80 per cent of the population born in 1910 or later were reported to be vaccinated with 3 doses of inactivated vaccine In 1963 a fourth dose, given to persons vaccinated 4 to 5 years earlier, was included in the general vaccination program The morbidity rate of paralytic poliomyelitis and the circulation of poliovirus in the community were markedly reduced (10, 21) In the years 1963 and 1964 no case of paralytic poliomyelitis was reported This study reports of the immunologic response and persistence of poliovirus antibodies induced by vaccination with inactivated vaccine It was examined by systematic controls in pre-immunization triple-negative vaccinees and by follow-up studies

MATERIAL AND METHODS

Imported inactivated poliovirus vaccine manufactured by Eli Lilly Pharmaceutics USA according to procedure outlined by *Salk* (24) was used for the majority of the two primary inoculations in 1957

gained from production and use of the vaccine The seed strains used were the Type 1 Stockholm 1423/53 (Stockholm 53) the Type 2 MEF 1 and the Type 3 Saukett strain Later the Type 1 Brunender s strain was introduced In the sequel some of the pertinent studies will be described

The antigenic poten

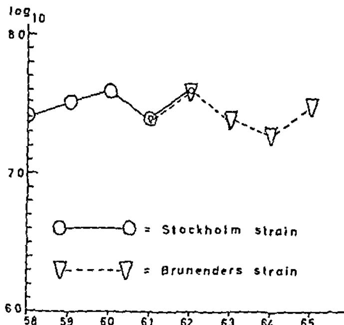


Fig 3

Mean level of titres of Type 1 poliovirus suspensions prior to inactivation The vertical axis gives the virus titre expressed as $\log_{10}$ $TCID_{50}$ per ml, the horisontal axis the year in which the vaccines were administered The small circle and triangle indicate that means were calculated from 6 respectively 2 values only, the bigger circles and triangles all represent means of 12 24 values

as to contain as little inactivated virus as possible Thus undiluted virus suspension was inoculated into tissue cultures Excess virus was removed after a 2 hour adsorption period fresh medium was added and the cultures were harvested within 24 hours at 37° C.

Preparation of sera Blood samples were collected either by venipuncture or by [illegible] immediately mixed with 1 2 ml [illegible] nt heparin After centrifugation [illegible] removed and the samples were [illegible] ° C

Neutralization test The antigen was diluted to contain 4 $\log_{10}$ $TCID_{50}$ per ml, and 1 part of virus was mixed with 9 parts of serum diluted in five fold increments (1 5–1 625) The virus concentration during the virus serum reaction was thus 2 $\log_{10}$ $TCID_{50}$ per 0 1 ml The mixture was kept in a waterbath at 37° C for 6 hours and in a refrigerator at 4° C over night Rubber stoppered small bore tubes were used for this purpose in order to avoid unnecessary evaporation On the following day the mixture was diluted 1 5 and 0 05 ml aliquots were inoculated into tissue culture tubes Approximately 1 $\log_{10}$ $TCID_{50}$ of virus was thus inoculated into each tube In 1957 to 1960 two tubes per dilution were used, later 4 tubes were inoculated The virus control was treated similarly but the serum was substituted by Parker 199 medium The control virus was diluted 1 5, 1 50 and 1 500 and 0 05 ml of each dilution was inoculated into each of 10 tubes The cultures were examined for cytopathic changes usually on the seventh day Only those tests were included in the results in which the virus control showed 100 per cent cellular degeneration [illegible]

given as the titre

The pre vaccination samples in 1957 were tested according to the conventional neutralization test at that time, *i e* incubating serum dilutions with virus at room temperature for one hour

Methodological studies Three gammaglobulin solutions containing relatively high concentrations of poliovirus antibodies (1·3125–1 15625) were titrated 5 times each by 3–4 technicians The variations of the results of these titrations are listed in Table 1 A slightly higher variance "between" different technicians than "within" technicians is indicated. A standard, serially diluted gammaglobulin solution was tested throughout 82 sets of tests against all three types of poliovirus (*Table 2*).

TABLE 1

Variations of Antibody Titres to Poliovirus Type 1 of three Gammaglobulin-Preparations "between" and "within" the Technicians

Experiment 1 γ-globulin I				Experiment 2 γ globulin II			Experiment 3 γ-globulin III			
Technicians				Technicians			Technicians			
A	B	C	D	A	B	C	A	B	C	D
6	5	5	6	6	6	6	5	5	5	6
"	"	"	"	"	"	5	"	"	"	"
"	"	"	5	5	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	3	4	"	"

Each titration experiment was carried out on the same day, using the same batch of cell cultures Three to four technicians performed five replicate titration tests Titres are given as the reciprocal of the highest serum dilution (expressed in 5 logarithms) that neutralized at least half of the tubes inoculated

Relationship to British Standard Units Ten titrations with the British Standard poliomyelitis antisera (23), diluted in twofold steps resulted in a mean titre of 1 64 (1 8 log_{10}) to Types 1 and 2 and 1·128 (2 1 log_{10}) to Type 3

Cross neutralization tests between the two Type 1 strains used for vaccine production, i e the Stockholm 53 and Brunender strains, were performed by use of the immuno-inactivation method (The former strain was employed for the antibody tests, even after the introduction of the Brunender strain in the vaccine) No intratypic strain specific antigenic difference were found by use of the routine method

Plan of the study All vaccinations were performed during the spring and carried out according to the following schedule A blood sample was drawn, followed by a subcutaneous injection of 1 ml of inactivated vaccine Two to three weeks later a second injection was given A blood sample was taken 14 days after the second dose A third blood sample was collected approximately 8 10 months later Simultaneously, a third injection was given A fourth blood sample was drawn two weeks after the third dose From the groups vaccinated in 1957–58, 1958–59 and 1959–60 a fifth blood sample was collected 4 years later, in 1961, 1962 and 1963 respectively The recorded data are based on groups comprising between 72 to 164 children (numbers are given under legends of the figures)

Only sera from triple-negative children, i e children without demonstrable anti

TABLE 2

Variation of Poliovirus Antibody Titres of a Gammaglobulin Preparation Tested Repeatedly during a 2¼ Year Period

Period	No. of tests	Distribution of antibody titres								
		Type 1			Type 2			Type 3		
		1 2500	1 12500	1 62500	1 2500	1 12500	1 62500	1 2500	1 12500	1 62500
Dec 60–Aug 61	20	2	17	1	0	18	2	0	18	2
Sept 61 Aug 62	46	7	39	0	5	40	1	1	43	2
Sept 62 April 63	16	2	14	0	3	13	0	0	16	0
Total	82	11	70	1	8	71	3	1	77	4

The serial dilutions of the gammaglobulin were prepared in large volumes at the beginning of the test period, each dilution being dispensed in a number of small tubes and stored at −25° C. At each test one tube of each dilution was thawed and mixed with the test virus. The figures in the table represent the number of tests giving the titre indicated.

RESULTS

Immunization with three doses of inactivated vaccine The first diagram (upper part Fig 4) illustrates the antibody response to the vaccinations carried out in the spring of *1957* (2 doses) and *1958* (1 dose) Twentyfive per cent of the 7–10 year old vaccinees tested lacked demonstrable antibodies to all three types of poliovirus prior to vaccination As mentioned above only the triple negatives were further investigated The curves in the diagram represent accumulated frequencies of titre values (Thus the value on the ordinate corresponding to the value 1 on the abscissa represents the percentage of children with demonstrable antibodies in undiluted serum or higher dilutions the value at 5 those having titres of 1 5 or higher, etc) After two injections 90 per cent of the children had demonstrable antibodies to *Type 1* poliovirus in the undiluted serum Fifty per cent of the vaccinees were estimated to have antibody titres of 1 8 or higher These 50 per cent i e median values will be used for comparative purposes A decrease of titres on an average fourfold was found at the end of the following 7 month period After the third injection 93 per cent became seropositive and 50 per cent reached levels of 1 95 or higher

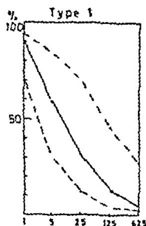
The antibody response to *Type 2* was significantly higher both after the two primary injections and the booster dose After two injections the median value was 1 62 After three injections all were seropositive and over 80 per cent reached titres of 1 625 or higher No apparent fall of antibody titres was observed during the period between the second and third dose

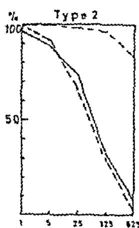
The response to *Type 3* was rather similar to that to Type 1 After two injections 50 per cent reached levels of or above 1 28 and after three injections the corresponding level was 1 58 The conversion rate from seronegatives to seropositives was 93 per cent

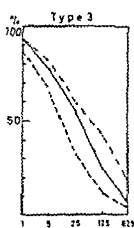
In the second study carried out in *1958–59* the vaccine was tested on triple negative schoolchildren 14–15 years of age (middle part of Fig 4) Eight per cent of these vaccinees were found to be triple negative prior to vaccination The antibody response to Types 1 and 2 appeared almost unchanged compared to the results of the preceding year The antibodies were not titrated beyond 1 625 (2 8 $\log_{10}$) however and thus especially the end points of the apparently very high Type 2 antibody levels after the booster injection could not be evaluated The response to Type 3 increased significantly 100 per cent were seropositive and 50 per cent reached titres of at least 1 300 (2 5 $\log_{10}$) after two doses

In the following year *1959* the sera from 7 to 10 year old school children were tested (lower part Fig 4) Twentyseven per cent were found to be triple-negative prior to vaccination In general the pattern of antibody response did not deviate from the preceding year although a slight increase of titres against all types was noted

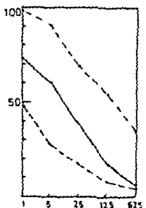
1957 – 58

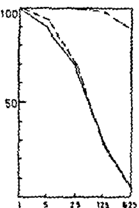


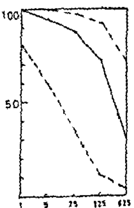




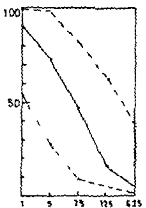
1958 – 59

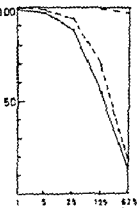






1959 – 60





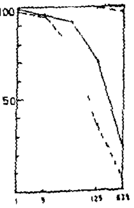


Fig 4

From the year *1960*, when 1–2 year old children were tested blood samples were collected by finger puncture and immediately diluted as described earlier Thus lower serum dilutions than approximately 1 10 could not be tested When comparing Figures 4 and 5, the difference in scale on the abscissa must be kept in mind Ninety five per cent of the children tested in that year were found to be negative to all three types of poliovirus at a serum dilution of 1 10 The pattern of antibody reaction typical of the two preceding years was repeated (Fig 5, upper part)

The results of the vaccination of children of 1 year old in *1961* (Fig 5 middle part) indicated for the first time a considerably increased antigenicity of the Type 1 poliovirus component All children tested had demonstrable antibodies in the dilution 1 50 (1 7 $\log_{10}$) after the booster and almost 70 per cent had titres of 1 6250 (3 8 $\log_{10}$) or higher This stands in contrast to the preceding year, when only 23 per cent reached titres of at least 1 6250 The group vaccinated in *1962 63* responded with similar high titres to all three types of poliovirus (Fig 5, lower part)

For a comparison of the antibody stimulating capacity of the vaccines used, the median antibody titres after two injections are listed

TABLE 3

Median Poliovirus Antibody Titres of the Vaccines after 2 Injections

Values given as $\log_{10}$ of reciprocal titre

Year group	No of sera	Type 1	Type 2	Type 3
1957	69	0 90	1 79	1 45
1958	124	1 05	1 74	2 48
1959	162	1 24	2 18	2 37
1960	134	1 30	2 60	2 89
1961	100	2 24	2.2[illegible]	2 62
1962	74	2 07	2 66	2 71

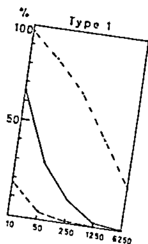
Fig 4

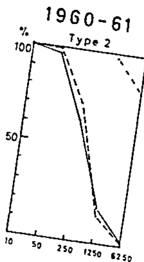
Upper row *Immunologic profiles of children vaccinated in 1957 58* Accumulated titer distribution of serum samples collected 14 days (solid lines) and 7 8 months (dashed lines) after the two first injections and 14 days after the third injection (dashed and dotted line) The vertical axis gives the accumulated percentage *i e* percentage of children attaining or surpassing indicated titre The horisontal axis givs the antibody titre expressed as the reciprocal of the last neutralizing serum dilution 14 day post primary vaccination samples (2nd samples) n = 69 7 8 months post primary vaccination samples (3rd samples) n = 87 and post booster samples (4th samples) n = 134)

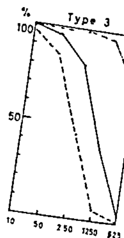
Middle row *Immunologic profiles of children vaccinated in 1958 59* 14 day samples *n* = 124 7 8 months samples *n* = 87 postbooster samples *n* = 72

Lower row *Immunologic profiles of children vaccinated in 1959–60* All profiles based on 162 samples

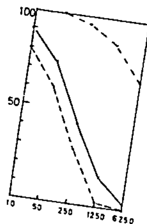
1960-61

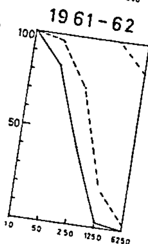


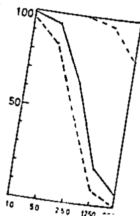




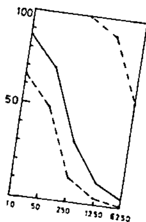
1961-62

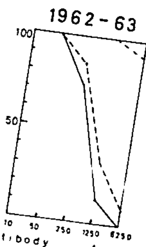


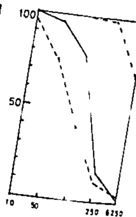




1962-63







accumulated percentage of vaccinees

antibody titre

——— 2nd samples
- - - - 3rd samples
— — — 4th samples

Fig 5.

in Table 3 When evaluating these figures it must be remembered that the test groups are not quite congruous and that a slightly different technique of blood sampling was used in the last three studies

To conclude the results with the individual types of poliovirus, the following will be mentioned

The *Type 1* virus induced a relatively poor antibody response during the years 1957–60 compared to that of Types 2 and 3 In 1961 and 62 a considerable rise in titres was observed

The *Type 2 responses* were constantly at a high level No apparent reduction of antibody titres between the second and the third dose was observed, a phenomenon that constantly occurred with Types 1 and 3 antibodies

The antibody response to *Type 3* was comparatively low in 1957 In the following years, however, the values increased, reaching levels equal to or higher than the response to Type 2

Persistence of antibodies The first follow-up study was carried out in the autumn of 1961 on the children immunized and tested in 1957–58 Similar studies were performed in 1962 and 1963 on children vaccinated in 1958–59 and 1959–60, respectively Fig 6 illustrates the antibody status of these children in 1961, 1962 and 1963, compared with their antibody levels 14 days after the third dose

A reduction of the antibody levels against all three types of polio virus occurred during this 3½-year period after the vaccination The median titre levels to Type 1 were reduced by 1 42 $\log_{10}$, 1 11 $\log_{10}$ and 1 26 $\log_{10}$ between 1958–61, 1959–62 and 1960–63, respectively The Type 2 antibody reduction could not be evaluated properly, as the end-point titres after the booster injection were not reached within the range of dilutions tested These remained on higher levels than the other two types after the three-year period

The median titre to Type 3 was reduced by 1 53 $\log_{10}$ between 1958 to 1961 The fall in titre to Type 3 of the second and third groups, *i e* between 1959 to 1962 and 1960 to 1962 could not be calculated accurately as the majority of the initial post-vaccination titres probably exceeded the dilutions tested After a 3½ years period they still had

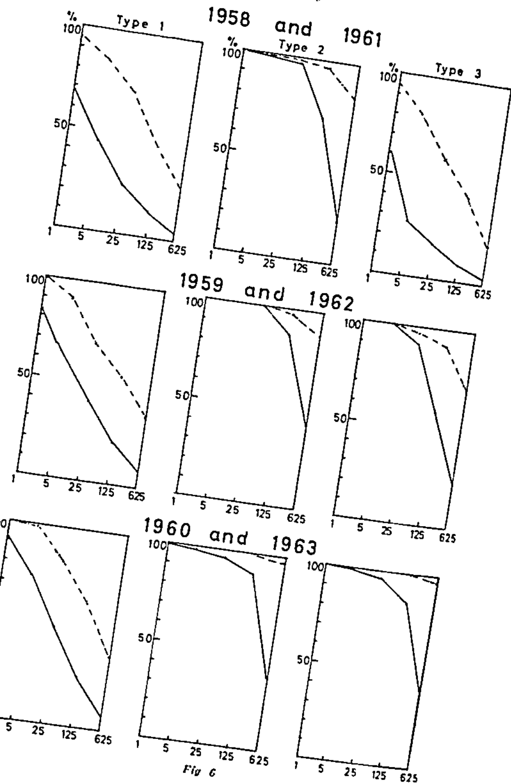
---

Fig 5

Upper row *Immunologic profiles of children vaccinated in 1960–61* Symbols as in fig 4 Observe that serum dilutions are 1 10 1 50 etc All profiles" based on 136 samples

Middle row *Immunologic profiles of children vaccinated in 1961–62* 14 day samples n = 100 7 8 months samples n = 80 14 day postbooster samples n = 79

Lower row *Immunologic profiles of children vaccinated in 1962–63* 14 day samples n = 74 7 8 months samples n = 86 14 day post booster samples n = 80



1958 and 1961
Type 1
Type 2
Type 3
%
100
50
1
5
25
125
625
1959 and 1962
1960 and 1963

Fig 6

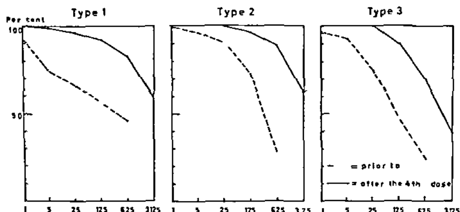


Fig 7

Effect of booster injection given 5 years after primary immunization
The solid line shows the antibody status immediately prior to and the dashed line the status 14 days after the booster

a 50 per cent level above 1 100 (2 0 $\log_{10}$) and all children had demonstrable antibodies in the dilution 1 5

The booster given 4 years after the primary vaccination raised the median antibody titre by 1–2 $\log_{10}$ units (Fig 7)

DISCUSSION

Antigenicity of poliovirus Type 1 As the antigenic potency of the Type 1 component in the poliovirus vaccine in this as in other laboratories presented the greatest problems, special attention was paid to its improvement A number of alterations of the vaccine manufacturing procedures were undertaken which were followed by improvement of the outcome of the antigenic potency test of the final product

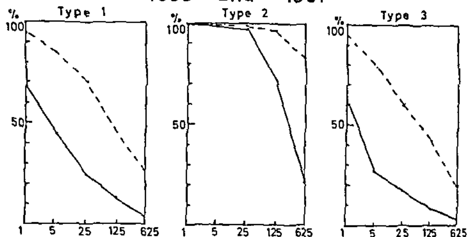
---

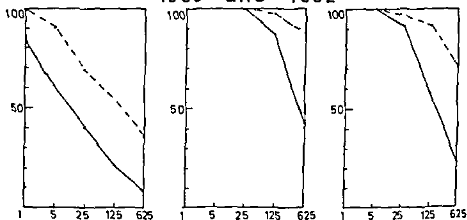
Fig 6

Upper row *Immunologic profiles of children vaccinated in 1957 58 14-day and 3 year post booster samples* Accumulated neutralization titer distribution of 14 day post booster serum samples (in 1958) is marked by a dashed and dotted line and of the 3 year post booster samples (in 1961) by a dashed line Both "profiles" are based on 134 samples

Middle row *Immunologic profiles of children vaccinated in 1958 59*
14 day and 3 year post booster samples
14 day post booster samples n = 72
2 year post booster samples n = 97

Lower row *Immunologic profiles" of children vaccinated in 1959–60*
14 day and 3 year post booster samples
14-day post booster samples n = 162
3 year post booster samples n = 98


1958 and 1961
%
Type 1
100
50
1
5
25
125
625
%
Type 2
100
50
1
5
25
125
625
%
Type 3
100
50
1
5
25
125
625


1959 and 1962
100
50
1
5
25
125
625
100
50
1
5
25
125
625
100
50
1
5
25
125
625

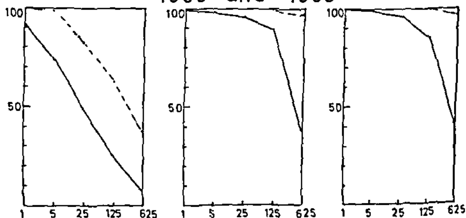

1960 and 1963
100
50
1
5
25
125
625
100
50
1
5
25
125
625
100
50
1
5
25
125
625

Fig 6

Antibody response Irrespective of the type of vaccine used and the age group vaccinated, a few phenomena reappeared throughout the study One was the constant fall of antibody titres of Types 1 and 3 in the interval between the second and third dose, while the Type 2 antibodies during this period appeared unchanged

As an explanation it might be assumed that this antigen is metabolized and eliminated at a lower rate than the others A prolonged antigenic stimulation would lead to relatively higher titres, the peak titre would probably not be reached within 2 weeks, which would explain the apparent stability of titres in the interval from 2 weeks to 7 months after the injections This problem seems worthy of further study

Persistence of antibodies As indicated by the three follow-up studies, there appeared a fall of antibody titres against all three types during the 3 year post-vaccination period The general antibody levels decreased by 1–1½ $\log_{10}$

Attempts to compare results from different laboratories and countries are rendered difficult by various factors such as laboratory techniques, heterogeneous test groups, epidemiological conditions etc However, the antibody stimulating capacity of the Swedish vaccines used during the first 4-year period appeared in general to be higher than that of the majority of inactivated vaccines used and reported at that period (11, 13, 15, 22, 26,) The last years' result, i e in 1961–62 and 1962–63, when 100 per cent of the triple-negative children obtained titres to all three types of poliovirus exceeding 1 50, appear to be equal to or even higher than the best results reported with purified and concentrated vaccines (6,11)

The fall of titres during the 3-year post-vaccination period is similar to that observed by others (2, 14, 25) Thus in a Danish study a mean fall of antibody titres in pre vaccination triple negatives amounting to 1 1, 0 9 and 1 3 $\log_{10}$ for Types 1, 2 and 3 respectively was found 2½ years after the booster injection (14) The Type 3 antibody fall was slightly more pronounced compared to the other two types This phenomenon can be recognized in the present study as well as in follow-up studies of vaccinations with live vaccine (3)

Effect of booster injection after four years The booster injection given 4–5 years after the first immunization appeared to bring the antibody titres up to the same level as after the previous booster (cf Figures 6 and 7) According to experience with repeated booster injections of other protein containing antigens (28) and with individuals with natural immunity to poliomyelitis infection (4), who have probably had repeated contacts with the antigen, one might expect a less marked titre fall after several boosters

Effect on morbidity ratio and prevalence of poliovirus When evaluating the efficacy of the immunization with the inactivated vaccines the comparatively low frequency of naturally occurring immunity in

the population must be considered. These circumstances were inves-tigated during the years preceding the initiation of general vaccination (16, 19, 20). In particular the continously increased morbidity rate among young and older adults was indicative of a population highly susceptible to poliovirus infection (19).

During the 20-year period before the introduction of poliovirus vaccination the number of paralytic cases was on an average 1136 per year (17 per 100,000). Since vaccinations started in 1957, altogether 431 cases have been reported. In 1963 and 1964 no case was recorded. The efficacy of protection was especially well elucidated in a study of an epidemic in Gothenburg in 1961 (1), where it was calculated to be 98 per cent against paralytic poliomyelitis with persisting paralysis.

How long this protection will last must be left an open question as systematic revaccinations have been carried out 5 years after the primary vaccination. All that can be assumed now is that a parenterally induced immunity of a similar magnitude to that demonstrated in this study, in a community with comparatively low natural immunity will with a very high degree of certainty protect vaccinees from paralytic poliomyelitis with sequelae for at least a five-year period.

Another consequence of the vaccinations appeared to be an almost complete eradication of circulating poliovirus in the community. Isolations of poliovirus in the laboratories were markedly reduced during the last 5 years, although the number of stool samples increased. In 1963, 1964 and 1965 two strains of poliovirus were isolated in Sweden from more than 6000 stool samples investigated yearly at the different virus laboratories. These two strains were to a high certainty directly imported.

In an experiment carried out by *Horstmann* and coworkers (12) in an earlier phase of poliovirus vaccination, it was stated that parenterally induced immunity did not inhibit natural infection and replication of wild poliovirus. However, *Gard* and coworkers (9) observ[ed] a relative resistance to infection, dependent upon the actual antibo[dy] titre at the time of exposure. *Dick* and coworkers (5) later showe[d] that if the antibody response to the parenteral vaccination reache[d] level higher than approximately 1:2200, there appeared a markedly lower rate of virus excretion.

The experience gained in Sweden after general vaccination with only inactivated vaccine provides strong support for these observations.

SUMMARY

The antibody response of triple-negative children to vaccination with Swedish inactivated vaccine was followed during the years 1957 to 1962, i. e. a period during which approximately 80 per cent of the population born in 1910 or later was successively vaccinated and the morbidity rate was reduced from a mean rate of 17 per 100 000 to

almost zero Wild circulating poliovirus appeared completely depressed The antigenicity of the Type 1 component, which in the Swedish as well in other vaccines was the weakest, appeared improved after change of the seed strain from Stockholm 53 to the Brunender s strain and introduction of a modified inactivation technique The last years investigations indicated that all childen developed antibody titres to all three types of poliovirus of 1 50 or higher

REFERENCES

1 *Andén T & Lycke F* An outbreak of poliomyelitis in a partially vaccinated population Acta path et microbiol scandinav 57 75–80 1963

2 *Brown G C* Duration of immunity Fifth Int Poliomyelitis Conf Copenhagen 1960 pp 146–154 (J B Lippincott Co)

3 *Buser F Fleury C & Rohner E* Status of immunity 5 years after the oral vaccination against poliomyelitis Ninth symp Europ assoc poliomyelitis and allied dis Stockholm pp 129 137 1963

4 *Böttiger M Gard S & Lagercrantz R* Vaccination with attenuated poliovirus Type 1 the Chat strain I A study in 20 families Acta Peadiat Scand In press

5 *Dick G R A Dane D S McAllister J Briggs M & Nelson R* Vaccination against poliomyelitis with live virus vaccines Effect of previous Salk vac

6

7

pigs in test for immunogenic capacity of poliomyelitis in virus preparations Arch Virusforsch 6 401–411 1956

8 *Gard S* Immuno inactivation by poliovirus Arch Virusforsch 7 449 460 1957

9 *Gard S Bottiger M & Lagercrant R* Vaccination with attenuated poliovirus

10

11

12

vaccinated and naturally immune humans to ingestion of attenuated poliovirus Tr Assoc Am Physicians 70 91 101 1957

13 *Kokko U P & Murray R* Human and animal antibody responses to commercial poliomyelitis vaccine Bull Wld Hlth Org 22 263 272 1960

14 *von Magnus H* Present knowledge about duration of sero immunity after vaccination with inactivated poliovaccines Fifth Int Poliomyelitis Conf Copenhagen 1960 pp 155 156 (J B Lippincott Co)

15 *Murray R* The standardization of potency of poliomyelitis vaccine Fifth Int Poliomyelitis Conf Copenhagen 1960 pp 176–185 (J B Lippincott Co)

16 *Melén B Wrange G & Olin G* Antibodies to poliomyelitis in school children in Stockholm Arch Virusforsch 4 437 445 1958

17 *Melén B Wrange G & Salenstedt R* Experiences with guinea pig test for immunogenic capacity of poliomyelitis virus and virus preparations Arch Virusforsch 7 369–377 1957

18 *Melén B* Potency of inactivated poliovirus vaccines An *in vitro* method of assay of antibody combining capacity Arch Virusforsch 9 135–149 1959

19 *Olin G & Heinert V O* The epidemiological pattern of poliomyelitis in Sweden 1925–1944 Acta path et microbiol scandinav Suppl 91 pp 139 151 1951

20 *Olin G & Wesslén T* Seroimmune patterns for poliomyelitis in Sweden Arch Virusforsch 2 191 205 1957

21 *Olin G* Protective efficacy of inactivated poliovirus vaccines Swedish experiences Fifth Int Poliomyelitis Conf., Copenhagen 1960 pp 114 119 (J B Lippincott Co)

22 *Perkins F T & Tobin I H* Antigenic activity of British poliomyelitis vaccine Brit Med J vol i pp 366–368 1957

23 *Perkins F T & Evans D G* British standard poliomyelitis antisera Types 1 2 and 3 Brit Med J vol i pp 1549 1553 1959

24 *Salk J E* Poliomyelitis vaccine in the fall of 1955 Am J Publ Hlth 40 1 4 1956

25 *Salk J E* Persistence of immunity following administration of formalin treated poliovirus vaccine Fifth Int Poliomyelitis Conf., Copenhagen 1960 pp 157 175 (J B Lippincott Co)

26 *Skovranek V* Investigations of the effectiveness of inactivated vaccine against poliomyelitis in Checkoslovakia. Fifth Int Poliomyelitis Conf., Copenhagen 1960 pp 124–137 (J B Lippincott Co)

27 *Wesslén Lycke E Gard S & Olin G* Inactivation of poliomyelitis virus by formaldehyde Arch Virusforsch 7 125–135 1957

28 *Wilson G S & Miles A A* Topley and Wilson s principles of bacteriology and immunity Vol. II 1347 1964

Acta path et microbiol scandinav 66 257–261, 1966

The Department of Bacteriology, County Hospital Sundsvall Sweden
(Head E. Hammarström M.D.)

DIAGNOSIS OF ASYMPTOMATIC MENINGOCOCCAL CARRIERS BY A SELECTIVE MEDIUM

By

SVEND OLE ROEPSTORFF and ERNST HAMMARSTRÖM

Received 21.viii.65

For a long time periodical epidemics of meningitis have been studied with reference to the carrier rate of Neisseria meningitidis in the upper respiratory tracts. Although the techniques are not difficult in themselves, routine diagnostic tests have not come into general use. The main reason for this is the difficulty of isolating meningococci due to contamination with normal flora. This has greatly contributed to the fact that the meningococcus is so seldom isolated from routine swabs, giving an illusion of low carrier rates in a normal population compared with the relatively high carrier rates that have been found in previous surveys in special groups such as recruits.

U. Berger (1) showed that a selective medium for isolating Neisseria catarrhalis, containing ristocetin, was effective in inhibiting gram positive cocci and mentioned "en passant" an increased count of meningococci.

Thayer & Martin (5) reported on a selective medium for Neisseria meningitidis and Neisseria gonorrhoeae containing polymyxin and ristocetin. Pure cultures of gonococci and meningococci were tested against different concentrations of antibiotics, the highest concentration that allowed bacterial growth being used. A detailed account of the inhibitory effect was only given for the gonococcus. Regarding the meningococcus it was only mentioned that they grew well with the exception of group D, which occasionally was partly inhibited.

TECHNIQUE

Thayer and Martin's selective medium consisting of chocolate agar with 25 IU polymyxin/ml and 10 mcg ristocetin/ml (PR agar) has been used over the last 7 months of 1964 in the routine diagnosis of respiratory tract infections in the counties of Västernorrland and Jamtland. Thus all nose, throat and sputum specimens were inoculated on this selective medium, and to begin with comparative inoculations were performed on chocolate agar without antibiotics. Both media were incubated in an atmosphere of CO_2 at 37° C, and as a rule examined the next day.

The diagnosis was made on the appearance of colonies, oxidase positivity, Gram stain, fermentation on HAP medium (3) with dextrose, maltose, laevulose and in the majority of cases lactose and galactose, also inoculation of bloodagar at 20° C.

The samples were sent to the laboratory on ordinary agar slants within the hospital itself, and on Stuarts or Loeffler tubes when sent in by post The specimens were usually taken as ordinary nose and throat swabs without any guarantee that the swab had come into contact with the nasopharynx

MATERIAL

As mentioned above, all nose, throat and sputum specimens coming into the laboratory were inoculated on selective media, and this material was divided into two groups

Group (1) A survey of a total of 318 military personnel extending over the period 9th June 1964 to 18th December 1964 In almost all cases the intention was only to show the presence of the meningococcus In one instance the examinations were initiated by an actual case of epidemic meningitis in a recruit, and in the remainder the examinations were initiated by the occasional discovery of carriers

Group (2) A total of 2146 specimens which were taken over the period 20th August to 19th December 1964 In the majority of cases these were sent in without any suspicion of the presence of meningococci A proportion of the specimens originated in routine swabs from hospital patients who did not show any symptoms of upper respiratory tract infections, Others originated from examinations for staphylococcus aureus and haemolytic streptococci, usually from healthy people Only a minor proportion came from patients with clinical evidence of upper respiratory tract infections, similarly only a small proportion were due to contacts with meningococcal carriers

Group 2 is a fairly representative cross section of the civil population of the district

RESULTS

Compared with chocolate agar without antibiotics, the yield of meningococci on the PR-agar increased from 8 6 per cent to 37 1 per cent i e about 4 times (Table I) This increase was most pronounced in specimens which showed sparse growth of meningococci and in these cases the culture on chocolate agar most often proved negative (Table 2)

TABLE 1

Comparison between the Yield of Meningococci on the Polymyxin Ristocetin (PR) Agar and Ordinary Chocolate Agar

	no specimens	no positive	% positive
PR agar	70	26	37 1
Chocolate agar	70	6	8 6

TABLE 2

Comparison between the Yield of Meningococci and the Polymyxin Ristocetin (PR) Agar in Relation to the Number of Meningococci in the Specimens

	no of meningococcal colonies					total no of specimens
	—	(+)	+	++	+++	
PR agar	44	4	5	6	11	[illegible]
Chocolate agar	64			1	5	[illegible]

— no growth (+) +++ from several to many colonies

The inhibition of other organisms was very evident and the meningococci appeared most often in pure culture In many cases they occurred sparsely, and the diagnosis was not infrequently made on only a few colonies

All streptococci and all non pathogenic Neisseria with the exception of a proportion of Neisseria catarrhalis were totally inhibited This held true even for staphylococcus albus and aureus, excepting certain types of staphylococcus albus that were thought to have come from the genital tract originally (*e g* infants showing a simultaneous presence of coliform bacteria)

In contrast to the PR agar, the ordinary chocolate agar plates were heavily contaminated with normal flora The meningococci were difficult to detect, and without concentrating especially upon them they were easy to miss Only occasionally were they lying separately, so that subculture could be made without contamination

The meningococcal carrier rate in military units varied between 16 per cent and 50 per cent with a mean value of 30 per cent (Table 3) The frequency, 4 18 per cent, with a mean value of 11 per cent among the civilian population was considerably lower (Table 4) Over the period of these surveys, for different weeks or months obvious differences in frequences were not observed

TABLE 3

Meningococcal Carrier Rate in Military Camps in two Counties during the Last 7 Months of 1964

camp		date 1964	no of persons	no of carriers	% carrier rate
I 21	Sollefteå	9/6	32	5	16
LV 5	Sundsvall	17/7	36	12	33
I 21	Sollefteå	15/9	14	7	50
I 21	Sollefteå	17/9	20	7	35
LV 5	Sundsvall	22/10	43	13	30
I 5	Östersund	6/11	36	9	25
I 5	Östersund	10/11	39	13	33
I 21	Sollefteå	26/11	27	7	26
I 21	Sollefteå	3/12	50	14	28
LV 5	Sundsvall	18/12	21	9	43
totals			318	96	30

100 strains were tested for sensitivity, using the disc method, against sulphonamide penicillin G streptomycin tetracycline and chloromycetin (Table 5) All strains were sensitive to sulphonamide, tetracycline and chloromycetin 5 strains showed a diminished sensitivity to penicillin (MIC 0 1 0 3 IU ml) 44 strains showed lowered sensitivity to streptomycin 3 of the sulphonamide sensitive strains were isolated from sulphonamide treated cases

TABLE 4

Meningococcal Carrier Rate in Civilians in two Counties during the Last 4 Months of 1964

date (week) 1964	no of persons	no of carriers	% carrier rate
20/9 – 26/9	92	11	12
27/9 – 3/10	96	14	15
4/10 – 10/10	89	10	11
11/10 – 17/10	180	13	7
18/10 – 24/10	117	13	11
25/10 – 31/10	253	11	4
1/11 – 7/11	336	33	10
8/11 – 14/11	282	25	9
15/11 – 21/11	145	25	17
22/11 – 28/11	165	30	18
29/11 – 5/12	159	16	10
6/12 – 12/12	132	15	11
13/12 – 19/12	100	12	12
totals	2146	228	11

TABLE 5

Sensitivity of 100 Meningococcal Strains to five Chemotherapeutic Agents

drug	sensitive	moderately sensitive	slightly sensitive	resistant
Sulphonamide	100			
Penicillin	95	5		
Streptomycin	96	42	2	
Tetracycline	100			
Chloromycetin	100			

DISCUSSION

PR-agar is according to our experience very suitable for routine diagnosis It is easy to read, the morphological diagnosis being made in a few seconds by means of a stereoscopic microscope PR-agar effectively inhibits the majority of the normal inhabitants and the common pathogens of the nose and throat Even single meningococci can easily be recognized after 24 hours incubation No inhibiting effect was observed on the size of the colonies In no case were the meningococci missed For these reasons, polymyxin and ristocetin, in the concentrations used, do not seem to have had any inhibitory influence on the meningococcal strains in this study

Due to these properties the PR-agar gave a greatly increased yield of meningococci compared with conventional chocolate agar without antibiotics The extra yield was especially noticeable on specimens with few meningococci while those on conventional agar were easily masked by the contaminating flora The four times greater yield in this survey

can be considered as a minimal value because a much more thorough examination of the less effective substrate was made than is normally the case

The relatively high frequencies of meningococcal carriers in military personal are well known from previous surveys from Sweden (*Korlof* (4)) and abroad (ref *Topley & Wilson* (6) and others) Similar frequencies have also been found in school children

Not so well known is the fact that a moderately high frequency (11 per cent in this survey) can occur at the same time in civilians

Meningococcal meningitis occurs in Sweden not as an epidemic in the sense of a large number of cases arising over a short period Sporadic occurrence is the rule, and so called secondary cases, or several simultaneous primary cases in a smaller group are unusual It can be presumed that the number of meningococcal carriers during the actual survey exceeded 40,000 out of a population of 400 000, but in spite of this, only 2 clinical cases of meningococcal meningitis occurred

With regard to the wide spread of infection amongst civilians, and the small proportion of carriers who develop meningitis isolation of carriers or prophylactic treatment with sulphonamides would only have minor influence on the frequency of meningitis

SUMMARY

A selective medium for the isolation of *Neisseria meningitidis* and Neisseria gonorrhoeae, after *Thayer & Martin*, containing polymyxin and ristocetin in ordinary chocolate agar (PR agar), has been tested over a period of seven months

The medium gave an yield of meningococci about 4 times higher compared with chocolate agar without antibiotics

318 military personnel were shown to have a carrier rate of 30 per cent, and on a fairly representative cross section of the civil population of 2146 persons a frequency of 11 per cent was found In spite of these high rates during the survey, only two cases of meningococcal meningitis occurred out of a population of 400,000

All of 100 strains tested were sensitive to sulphonamide, and 5 strains showed lowered sensitivity to penicillin G

REFERENCES

1 *Berger L* A Selective Medium for Neisseria Catarrhalis ZBL Bakt *183* 135–138 1961

2 *Branham S F* Serological Relationships among Meningococci Bacteriological Reviews *16–17* 175–188 1953

3 *Juhlin I* A New Fermentation Medium for N Gonorrhoeae HAP medium Influence of Different Constituents on Growth and Indicator Colour Acta path et microbiol scandinav *58* 51 57 1963

4 *Körlof B* Forekomst av meningokocker på två militarforband vintern 1955 Svenska Lakartidningen 52 1031 1033 1955

5 *Thayer J D & Martin J I* A Selective Medium for the Cultivation of N Gonorrhoeae and N Meningitidis Public Health Reports *79* 49 57 1964

6 *Topley & Wilson* Principles of Bacteriology and Immunity vol 2 1964

Acta path et microl iol scandinav 66 262 263 1966

BRIEF REPORT

THE ANTIGEN COMPOSITION OF ECHO TYPE 6 VIRUS STUDIED BY IMMUNODIFFUSION AND IMMUNOELECTROPHORESIS

By *Marianne Forsgren*

The antigen composition of ECHO type 6 virus has been studied by immuno liffusion

...

heating the antigen to 56° C with heated antigen (H) only one precipitate was fo...

pr...

co...

ha...

ge...

ty...

gave rise to a single line with native as well as with heated antigen concentrates The line obtained with the native antigen fused with the two precipitates N and H obtained with human sera and gave a red RNA fluorescence When compared with

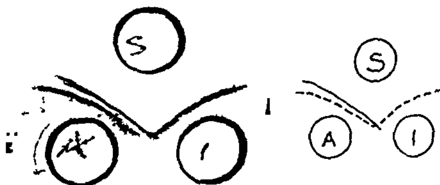


Fig 1

Agar double diffusion plate with ECHO type 6 virus precipitates stained with acridine orange A native ECHO type 6 virus concentrate containing N and spontaneous H antigen I concentrate A heated 30 min at 56° C containing only heated H S human convalescent serum containing N and H antibodies
—— N antigen antibody precipitate showing red fluorescence
H antigen antibody precipitate showing no fluorescence

Received 8 xii 65 from *the Virus Department of the Central Bacteriological Laboratory of Stockholm City*

the precipitate obtained with a human serum containing H antibodies only, a spur formation occurred Apparently, the native antigen guinea pig serum line represented two antigen antibody precipitates superimposed upon each other

Immunoelectrophoresis Native and heated (30 56° C) antigens were subjected to

arcs occurring at different distance from the origin Also at pH 7 0 heated and

References 1 *Beale J & Mason P J* J Hyg 60 113 121, 1962 —2 *Grasset F Bonifas V & Pongratz E* Proc Soc Exp Biol Med 97 72 77, 1957.—3 *Le B auter G L* Brit J Exp Pathol XL 452-463 1959 —4 *Ohlson (=Forsgren) M* Acta path et microbiol scandinav 57 494-495 1963 —5 *Ohlson (=Forsgren) M* Acta

[1] kindly supplied by Pharmacia AB Uppsala

Acta path. et microbiol. scandinav. 66, 264–265, 1966

BRIEF REPORT

HEREDITARY CYTOTOXIC FACTOR OF HUMAN SERUM

By S. Bergman

Cytotoxic factors in serum have long been studied (1, 2). In this investigation the cytotoxic factor of serum has been determined with the aid of a simple cell culture method (3, 4). After storage for 5 days at +4° C human sera could be classified by this method into one group in which the effect persisted (Cf+) and one in which it disappeared (Cf−) (5).

Methods. The experiments were performed on ordinary glassplates on which HeLa cells 100 000 cells/ml were sowed together with the serum to be tested. The serum was tested undiluted and diluted 1/2 and 1/4 in Hank's solution. After the cells had adhered to the glass for 6 hours they were allowed to grow for 48 hours and then fixed, stained and counted in regard to number of cells and frequency of mitoses (3, 5).

Results. Table 1 shows the results of the experiments with normal healthy adults.

Table 1. *Frequency of Cf Groups in Healthy Adults*

	Cf+	Cf−	n	Percentage Cf+
Men	28	15	43	65
Women	19	20	39	49
n	47	35	82	57

As is shown in Table 1 there is a slight predominance for the Cf+ group. An investigation of a family material is shown in Table 2.

Table 2. *Family Material*

	Mating		Children	
	Father	Mother	Cf+	Cf−
1	Cf+	Cf+	♂ ♂ ♀	—
2	Cf+	Cf+	♂ ♀ ♂	
3	Cf+	Cf+	♀ ♂ ♀ ♂ ♀	
4	Cf+	Cf−	♂ ♂	♀
5	Cf+	Cf	♂ ♂ ♂	
6	Cf−	Cf+	♀ ♂	♀
7	Cf−	Cf+	♀	♂
8	Cf−	Cf+	♂	♀
9	Cf	Cf+	♂ ♀	
10	Cf	Cf		♂ [illegible] ♀ ♂
11	Cf−	Cf		♀
12	Cf−	Cf	—	♀ [illegible] ♀ ♀

The family material shows that the Cf factor is hereditary.

Received 9.xii.63 from *Institute of Clinical Bacteriology, University* [illegible], Sweden.

This investigation is supported by grants from the Swedish Cancer Society.

Determination of Cf group was performed on a number of serum samples collected from patients with miscellaneous diseases (patients from the medical and surgical wards and from the department of dermatology) and especially from patients with rheumatoid arthritis. All samples were taken before treatment was instituted. The results are shown in Table 3.

Table 3 *Frequency of Cf Groups in Rheumatoid Arthritis and Miscellaneous Diseases*

	Cf+	Cf−	n	Percentage Cf+	χ^2 **
Rheumatoid arthritis	56	7	63	89	17.26
Miscellaneous diseases	23	15	38	61	0.11

* χ^2 is calculated on comparison with the material from normal persons described in Table 1.

Comparison of the results in Table 3 shows that in rheumatoid arthritis, which did not include cases of rheumatic fever or spondylarthritis, there is a significant difference ($p < 0.1$) in distribution of the Cf groups from normal distribution in the material of serum from healthy persons.

Discussion. Determination of Cf group was performed on a number of serum samples collected from healthy subjects on different occasions over a period of three years. The samples from any given individual were all of the same Cf group. There was no significant difference in the sex distribution of the two Cf groups (χ^2 2.2). The Cf factor is hereditary and the observations are consistent with the assumption of autosomal unifactorial inheritance.

Summary. The cytotoxic factor was determined on serum samples from healthy subjects in a family material and miscellaneous diseases.

The cytotoxic factor was shown to be hereditary and the distribution in rheumatoid arthritis differs from normal distribution.

References. 1. *de Luca C., Rakowski P. F. & Tritsch C. L.:* Biochim. Biophys. Acta 86 346–354 1964.—2. *Terasaki P. I. & Chamberlain Ch. O.:* J. Exp. Med. 115 439–452 1962.—3. *Bergman S.:* Acta path. et microbiol. scandinav. 59 279–291 1963.—4. *Bergman S. & Nilsson S. B.:* Acta path. et microbiol. scandinav. 59 161–164 1963.—5. *Bergman S.:* Cancerforskning, in press.

Acta path et microbiol scandinav 66, 266-277 1966

TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meetings April 10, September 25, and November 26-27, 1965

Received 21.xii.65

Meeting April 10, 1965

Gösta T Hultquist BRAIN DAMAGE IN CHILDREN AFTER DEEP HYPOTHERMIA FOR OPEN HEART SURGERY

Severe brain damage has been encountered in five children who died 1½-102 days after the use of deep hypothermia for open heart surgery

The brain changes consisted mainly of diffuse degeneration and/or disappearance of nerve cells and gliosis In four cases the changes were localized to the globus pallidus In three of these cases extrapyramidal symptoms appeared a few days after the operation and progressed slowly In one case the changes were localized to the cerebral cortex the hippocampus and cerebellum while the globus pallidus showed minor changes No neurological symptoms were observed in this child but it died very soon after the operation

The cause of the brain changes seems to be divergent in different cases but deep hypothermia was assumed to be important Changes dominating in the globus pallidus are usually ascribed to hypoxaemic hypoxia and cortical and cerebellar changes to oligaemic hypoxia

In the mechanism of the brain damage among other things vasomotoric disturbances aggregation of erythrocytes or thrombocytes paralysis of enzyme systems and changes of the blood brain barrier induced by deep hypothermia have been considered No conclusion is possible either concerning the mechanism of the brain damage or why children are especially susceptible to deep hypothermia

F Linell SYMPOSIUM ON HISTO PATHOLOGICAL DIAGNOSIS ON FUNGUS DISEASES

A Aarstad ACTIVE TUBERCULOSIS IN AN AUTOPSY MATERIAL

S Carlsson & O Grøntoft HISTOLOGICAL CLASSIFICATION OF PROSTATIC CARCINOMA

A Anberg CHANGES OF THE ULTRASTRUCTURE OF THE LIVER IN ICTERUS OF PREGNANCY

S Holm J Jónsson C E Zettergren RABBIT GLOMERULO NEPHRITIS INDUCED BY STREPTOCOCCI

K E *Astrom* CHANGES IN THE NERVOUS SYSTEM IN A ONE YEAR AUTOPSY MATERIAL

L *Angerwall* Y *Olsson* P *Sourander* & J *Save Söderbergh* CHANGES OF THE VESSELS AND THE PARENCHYMA OF THE NERVOUS SYSTEM IN JUVENILE DIABETES

A *Brun* THE FORMATION OF MARGINAL GLIO NEURAL HETEROTOPIAS

K *Kristensson* & P *Sourander* HISTOCHEMICAL STUDIES ON INFANTILE AND JUVENILE AMOUROTIC IDIOCY

O *Grontoft* THE PERMEABILITY OF P^{32} IN THE BRAIN IN NEW BORN AND OLD RABBITS IN SHORT TERM EXPERIMENTS

G *Ingvarsson* TRIALS WITH L DOPA ON DEPRESSION OF ENDOGENOUS ORIGIN AND ITS EFFECT ON ASTHMATIC BRONCHITIS

Meeting September 25, 1965

S *Falkmer* KERATOSIS FOLLICULARIS DARIER EHLERS DANLOS SYNDROME AND LIPOGLYCOPROTEINOSIS URBACH WIETHE

The Institutes of Pathology in Umeå receive all the surgical and autopsy specimens from all the hospitals in the Northern part of Sweden It was therefore considered worth while presenting some facts about the pattern of diseases in this area

Apart from the well known higher frequency of some infectious diseases the most striking difference in the pattern of diseases to that in the Southern part of Sweden is the high frequency of unusual hereditary diseases Families with fructose in tolerance malabsorption of glucose and galactose Gaucher s disease juvenile nephronophtisis pseudoxanthoma elasticum homocysteinuria and oxalosis are known Most of the cases of some other rare disease such as Sjogren Larsson s syndrome (ichtyosis spasticity and mental retardation) infantile hereditary agranulocytosis and keratodermia palmaris et plantaris have been reported from this region

Some families have been found in which keratosis Foll cularis Darier and Ehlers Danlos syndrome respectively have appeared in several generations These diseases seem to be dominantly inherited in contrast to Lipoglycoproteinosis of which several cases in 4 families have been found Detailed studies of the diseases are in progress

The reason why diseases of this kind are concentrated in this region may be that isolated communities with poor living conditions occur in this vast area

T *Arwill* & A *Bergenholtz* EPIDERMOLYSIS BULLOSA

In the Province of Vasterbotten 10 cases of the lethal form of *epidermolysis bullosa hereditaria* have been recorded A genealogic study has shown paternal and maternal consanguinity in 5 cases with a common ancestor in the beginning of the 17th

century Histological and electron microscopic studies on skin and mucous membranes show intercellular oedema in the Malpighian layer, vesicle formation collagenolysis in the connective tissue and destruction of the basal membrane

A trait which is constant in the lethal form is a poor mineralization of the enamel in form of hypoplasias and hypomineralization similar to the picture in *amelogenesis imperfecta* In demineralized sections of non erupted teeth squamous metaplasia of the enamel consisting of a conglomerate of epithelial *débris* partly mineralized and partly keratinized globular situated in vacuoles The ameloblasts seem to lose their mineralizing power at the onset of the mineralization of the enamel

The authors suggest that enamel dysplasia should be added to the symptom complex in *epidermolysis bullosa hereditaria letalis It can be seen clinically only if the patients survive the tooth eruption as in one of our cases who survived for* 13 months

T Angstrom & E Frisell FAMILIAL LETTERER SIWE DISEASE

Since 1951, when *Reese & Levy* reported two cases of familial Letterer Siwe disease there have been reports of identical or similar cases in about ten families under different names familial Letterer Siwe disease (*Falk* 1957, *Schoeck* 1963) familial haemophagocytic reticulosis (*Farquhar* 1952) generalized lymphohistiocytic infiltration (*Nelson* 1961) and familial erythrophagocytic lymphohistiocytosis (*MacMahon* 1963)

We have investigated a family with two children dying from a disease which clinically and histologically resembles Letterer Siwe disease but can be differentiated from leukaemia malignant lymphomas including Hodgkin s disease and congenital granulocytopenias The children a boy and a girl fell ill in early infancy with fever, infections anaemia granulocytopenia with lymphocytosis skin eruptions enlargement of liver spleen and lymph nodes Autopsy revealed *proliferation of* atypical lympho histiocytic cells including large macrophages in many organs Family investigation revealed consanguinity between the parent families nine generations earlier There is a third child in the family, a 6 year old boy now healthy who in infancy showed anaemia susceptibility to infections and transient hepato splenomegaly Examinations of bone marrow and peripheral blood in relatives showed pathological findings in the father some of his siblings and the grandfather

P Geertinger THE PARATHYROIDS IN SUDDEN UNEXPECTED DEATH IN INFANCY (SUD)

The parathyroids were examined in 122 consecutive legal autopsies on infants aged 2 11 months (chiefly from the University Institute of Forensic Medicine Copenhagen) In 82 cases the negative results of histological bacteriological and virol gical examination as well as the anamnestic data indicated typical SUD Forty cases (age 2 11 months) with clearly demonstrated causes of death (homicide accident etc) served as controls

By histological serial sections the parathyroids were found and normal in all cases but 3 in the control group In a few cases (aberrant) thymus tissue was found in the neighbourhood but *fusion* of this tissue with the parathyroids never occurred

In the group of SUD the parathyroids were not found in 28 cases In 54 cases the parathyroids were found but many variations in localization and morphology were observed. Thus in 32 cases masses of thymus tissue occurred *within the para*

thyroid capsule in 17 cases the two tissues being totally fused Parathyroid tissue never occurred in the thymus organ itself

The results indicate that SUD might be connected with incomplete development of the parathyroids the cause of which probably is to be found early in foetal life (4th to 6th week) This theory would explain the fact that SUD shows a marked seasonal fluctuation the same being true of parathyroid function

I M Turnbull A Breig & O Hassler BLOOD SUPPLY OF CERVICAL SPINAL CORD IN MAN A MICROANGIOGRAPHIC STUDY

Forty three cervical spinal cords were prepared by perfusing a vertebral artery in the cadaver with a fine grained barium solution These specimens were examined under a dissecting microscope and by microangiography

The number of cervical anterior radicular arteries varied from one to six posterior radicular arteries from zero to eight Four specimens did not show an anterior radicular artery on a C7 C8 or T1 root and this may explain some of the softenings of the central part of the lower cervical cord that follow mid cervical lesions

When the anterior spinal artery is solitary, 10 to 20 per cent of the central arteries that arise from it start on a short stalk which divides in the sagittal plane into two branches one for each half of the cord These common stems have not been recognized in the past because their bifurcations can not be visualized in transverse sections

The overlapping of arterial branches was studied in the longitudinal and trans verse planes and was found to be extensive The white matter is supplied from the periphery but its inner quarter to third also receives branches from the central arteries

Arteries on and within the cervical cords of many elderly subjects were tortuous and looped.

A P Baker F Bergman & K G Paul STUDIES ON HORMONAL CONTROL OF EOSINOPHILS IN THE UTERUS OF RODENTS

The regulatory effect of estrogen and progesterons as well as of degranulation of uterine mast cells on the cyclic variation of peroxidatic activity in the uterus accounted for by the presence of eosinophils was investigated

Data from the literature have shown that in estrogenprimed ovarectomized mice an increase in the enzyme level occurs Free progesterons almost completely in hibited the peroxidatic response to the short acting 17β estradiol but depressed only the initial response in regard to the enzymatic activity to the depoestradiol 17α hydroxyprogesterone capronate having a much longer duration of action than the free progesterone inhibited for a longer period of time the development of the peroxidatic activity induced by the depoestradiol

In addition to producing eosinophilia estradiol reduces the number of uterine mast cells according to the literature A possible inter relationship is suggested The uteri of ovarectomized rats given 48 80 according to the method of Riley, were devoid of eosinophils and essentially devoid of intact mast cells 6 10 days after the first injection of 48 80 Long term administration of 48 80 did not prevent the estrogen induced eosinophilia the estrogen was given four days after the first injection of 48/80

T Öberg ISOTOP STUDIES OF THE MAXILLARY JOINT IN GUINEA PIGS

B Persson & S Falkmer ELECTRON MICROSCOPY OF BRONCHIAL CARCINOIDS

The ultrastructure of bronchial adenomas of carcinoid type was studied in three cases Two were malignant with metastases to skeletal muscle and pleura respectively None was argentaffine The carcinoid syndrome did not occur in any of the cases

The ultrastructure of all the cases was similar and conformed well with that recently described by *Bouteille et al* (1964) The nuclei were uniform in size moderately electron dense with a prominent nucleolus and a marked nuclear membrane and perinuclear space The electron density of the ground substance of the cytoplasm varied A large number of mitochondria were observed and many appeared swollen with barely visible cristae The Golgi apparatus was prominent in most cells Likewise cytosomes and cytosegresomes were common and large Some multivesicular bodies were found The endoplasmic reticulum was both of smooth and rough type We also found small secretion granules always enclosed within a single layered sac and often showing a large space between the granule and the sac membrane In some cells these granules seemed to originate from the Golgi apparatus

These ultrastructural features of bronchial carcinoids are similar to those found in both gastro intestinal carcinoids and Kultschitsky cells and thus favour the view that bronchial adenomas of carcinoid type are hormonally active

L Bjersing THE ULTRASTRUCTURE OF CORPUS LUTEUM OVARIAN FOLLICLES AND ISOLATED GRANULOSA CELLS

Young active corpus luteum tissue and mature ovarian follicles from pigs were studied by electron microscopy In addition isolated porcine granulosa cells incubated with androstenedione in isotonic Krebs Ringer $NaHCO_3$ buffer with glucose for 1½ hour at 37 5° C were studied The main goal was to see whether morphologic signs of steroid hormone synthesis occurred in the granulosa cells

All the cells were largely alike with regard to the frequency and main ultrastructural characteristics of lipid droplets mitochondria and endoplasmic reticulum (ER) The ER was mainly agranular and fairly distinct In the corpus luteum cells several Nebenkerns or parasomes were found composed of concentrically arranged agranular ER As it is known that steroid producing cells usually contain much agranular ER besides hormone converting enzymes in the microsome fraction it seems probable that the parasomes of the corpus luteum indicate active steroid hormone synthesis

Some parasomes also occurred in the isolated granulosa cells This finding or at least the abundant agranular ER itself can be regarded as a morphologic sign of steroid hormone production in the granulosa cells The presence of lipid droplets and the structure of the mitochondria provide additional support

Meeting November 26–27, 1965

T Saldeen EXPERIMENTAL INVESTIGATION ON FAT EMBOLISM AND INTRAVASCULAR COAGULATION

The purpose of the investigation was to study 1) if an intravascular coagulation occurs in the lungs in pulmonary fat embolism and 2) the significance of such an intravascular coagulation

As there are no specific staining methods for intravital fibrin precipitations a new method for demonstration of fibrin in the lung was used J^{131} labelled fibrinogen was injected intravenously before the experiment Fat embolism was induced by bilateral femur fractures or direct intravenous injection of homogenized fat tissue in 44 rabbits and 315 rats A rapid uptake of the isotope in the lung was observed after fat injection The activity in the lung decreased to normal values in one hour Similar curves were seen after fractures Autoradiography showed that the activity was concentrated to the vessels

In animals pretreated with heparin or fibrinolysin no uptake occurred A fibrinolytic blocking agent counteracted the rapid fall of activity Injection of fat heated to 60° C for 15 minutes was not followed by any isotope uptake in the lung

In 225 rats fat was injected intravenously into the common carotid artery or intracardially It was found that pretreatment with heparin (5000 U/kg body weight) increased the tolerance towards the fat

G Voigt PTAH STAINING FOR DEMONSTRATION OF MYOCARDIAL LESIONS

B Larsson CONTUSIO PONTIS SEU REG BASALIS CEREBRI

It has been questioned if traumatic instantaneous hemorrhages occur in the brain stem and adjacent cerebral portions Among 44 consecutive legal cases 15 were accidents with central nervous system hemorrhages and signs of trauma adequate to have produced the hemorrhages 6 of these cases which expired immediately or within one hour revealed small similar hemorrhages in the brain stem and other basally located or more distal portions of the brain 2 cases with immediate death revealed only a moderate degree of brain swelling

One case which had survived unconscious for 9 days showed only pontine hemorrhages

The findings support the opinion that in severe head injury with preferable a parietal impact ponto basal hemorrhages appear immediately

B Larsson SPONTANEOUSLY HEALED TRANSVERSE RUPTURE OF THE AORTIC ARCH

A 43 year old man with a cardiac murmur since the age of nineteen but no symptoms of heart disease expired from uremia due to congenital polycystic kidneys There was no arachnodactyly and no history of trauma

At autopsy a circumferential aortic scar was found close to the attachment of the ligament of Botalli and the ostium of the left common carotid Between the sharp lines of the rupture there was a scar tissue appearing as a reinforced adventitia No other significant change of the aorta was found Traumatic etiology was considered probable

P Sourander H A Hansson K Kristenson Y Olsson & L Svennerholm TISSUE CULTURE STUDIES ON HEREDITARY METABOLIC BRAIN DISEASES

A Moberg FUNCTIONAL ASPECTS ON EXTRACARDIAL VESSEL ANASTOMOSIS (To be published in Acta Radiol)

I Hägerstrand & G Fichera THE SMALL LYMPH VESSELS OF THE LUNGS IN LYMPHANGIOSIS CARCINOMATOSA

J Save Soderbergh L Angervall S Enestrom & S E Fagerberg MORPHOLOGY AND PATHOGENESIS OF DERMAL MICROANGIOPATHY IN YOUNG DIABETIC MEN

Skin punch biopsy was performed in 38 diabetic men (mean age 31 1 ± 1 5 years) with varying durations of the disease and 17 nondiabetic men (mean age 31 7 ± 2 8 years) No significant difference was recorded in the mean systolic and diastolic blood pressures respectively between the groups

The changes in venules and capillaries as seen by light microscopy were arbitrarily graded twice independently by two of us with no access to clinical data A grading system with 5 degrees was used for assessing the wall thickness (w t)

The most characteristic lesion was thickening of the blood vessel walls Significant thickening was noted in 14 diabetics and 1 control Significant correlation between w t and endothelial proliferation was not found among the diabetics nor was there any difference between the groups The nuclei of the pericytes often seemed prominent comparatively large and hyperchromatic Positive significant correlation was shown between w t and this pericyte change in the diabetics but not in the controls In two selected diabetics with severe wall thickening 4+ and 5+ by light and phase contrast microscopy the capillary basement membrane thickening in electrone microscope was homogenous and or stratified

The w t was significantly and positively correlated to the age of the diabetics duration of diabetes and diastolic blood pressure Multiple correlation however only revealed a significant correlation between w t and the duration of diabetes This means that the demonstrated correlations to age and diastolic blood pressure can be accounted for by the dependence on duration of the w t In the control group no significant correlation was shown between w t and age or systolic or diastolic blood pressure Strong positive correlation was demonstrated between w t and retinopathy

The investigation indicates that w t is dependent of the duration of diabetes and implies that the pericytes may be involved in the development of dermal capillary and venule lesions in diabetes

L Konig ON THE MECHANISM OF GROWTH OF ROUS SARCOMA

B Stenkvist *IN VITRO* STUDIES ON HUMAN AND BOVINE CELLS TRANSFORMED BY ROUS SARCOMA VIRUS

Human and bovine cells exposed *in vitro* to Rous sarcoma virus RSV underg morphological changes similar to those exhibited by chick cells ex[illegible] in RSV *in vitro*

It has been demonstrated that human and bovine cells transformed by RSV as well as control cells have a limited life span in tissue culture in contrast to cells

transformed by SV 40 virus By clonal analysis of bovine cells transformed by RSV it was found that three types of cells were present in transformed mass cultures 1) morphologically normal clones with cells capable of at least 40 divisions 2) morphologically transformed cells with a tendency to degenerate 15–20 cell divisions and 3) morphologically transformed cells with capacity to revert to normal morphology and thereafter ability of the cells of nearly as many cell divisions as cells derived from normal clones

In addition to the morphological transformation of human and bovine cells induced by RSV contact inhibition is permanently abolished

The growth rate of transformed cultures is increased initially but after 25 passages it is the same as in control cultures

P Sundelin CYTOLOGICAL AND CYTOCHEMICAL *IN VITRO* STUDIES ON EMBRYONIC CHICK FIBROBLASTS AFTER INOCULATION WITH ROUS TUMOUR VIRUS

J Mark CHROMOSOMAL ANALYSIS OF ROUS SARCOMA IN MICE

L G Lindberg & P Biberfeld ROUS RAT SARCOMA STUDIED WITH FERRITINCONJUGATED ANTIBODIES

No virus particles have been observed in mammalian tumors induced by Rous virus The virus information however, is present in the sarcoma cells as retransfer of the mammalian tumor cells to the chicken elicits a Rous sarcoma with virus producing capacity

This problem was attacked in a previous study using the fluorescent antibody technique A specific fluorescence associated with the outer cell membrane of the rat sarcoma cell was found It was suggested that the rat sarcoma cell contained an antigen identical in both chicken and rat sarcoma cells

The present study was carried out on Rous rat sarcoma transformed into ascitic form by intraperitoneal injection of finely minced sarcoma tissue The ascitic fluid which appeared in the ensuing days contained numerous vital sarcoma cells They were incubated with anti Rous serum produced in chickens After thorough washing the sarcoma cells were then incubated with ferritinconjugated anti-chicken γ globulin serum from rabbits

Examination in the electronmicroscope of Epon embedded specimens revealed conglomerates of ferritin particles located just under the cell membrane Tagging of specific cell components or of structures resembling Rous virus particles was not seen The results suggest that the immune reactions are due to tumor specific antigens common to Rous chicken sarcoma and to Rous rat sarcoma They confirm earlier results obtained with the fluorescent antibody technique

P Biberfeld C Soderholm & P Perlmann ELECTRON MICROSCOPICAL STUDIES WITH PURIFIED FERRITIN LABELLED ANTIBODIES

During an investigation of polyoma virus antigen in infected culture cells use was made of the ferritin antibody technique (*Singer* 1959) Experiments with crude ferritin globulin conjugates indicated the necessity to remove free ferritin from the conjugates For this purpose column electrophoresis of ferritin conjugates on dextran gels (Sephadex) was tried

Although no complete quantitative separation was accomplished after electro-

phoresis for 16–20 h fractions were obtained which—after elution of the column—contained immunoelectrophoretically pure globulin ferritin conjugate Agar diffusion tests of these fractions showed patterns of partial identity between precipitin lines formed against anti ferritin and antiglobulin Antibody activity was retained after the purification procedure as shown by inhibition of virus haemagglutination with conjugated antipolyoma virus immunoglobulin

The immunochemical staining properties of purified conjugates were tested at the ultrastructural level Polyoma virus infected cultures were harvested the cells freeze sectioned and treated either with purified specific immunoglobulin conjugate or conjugated normal globulin Electron microscopical examination revealed specific tagging of polyoma virus with ferritin conjugated immunoglobulin, whereas preparations treated with normal globulin conjugate had much less ferritin within the cells and the ferritin granules were only randomly associated with virus particles.

B Lagerlöf THE HISTOGENESIS AND THE ALKALINE PHOSPHATASE ACTIVITY OF MICE THYMOMA INDUCED BY X RAY AND VIRUS

S Franzén P A Jacobsson J Zajicek & T Angström CYTOLOGIC DIAGNOSIS OF MAMMARY TUMOURS BY ASPIRATION BIOPSY

3150 aspiration biopsies of mammary tumours were carried out during the years 1955 1963 In 1666 of these cases (52 9 per cent) aspiration biopsy was followed by surgical treatment and the operation specimen examined histologically

In 89 2 per cent of 785 cases histologically reported as benign lesions aspiration biopsy gave no evidence of malignancy in 8 per cent cell atypias were observed and in 2 7 per cent the cytologic picture was suspicious for carcinoma 1 case was reported as carcinoma

30 cases were histologically diagnosed as precancerous lesions In 24 of these the cytologic diagnosis was negative for malignancy in 1 case cell atypias were observed 4 cases were reported as suspicious for carcinoma and 1 case as *carcinoma*

In 851 cases the histological examination revealed carcinoma In 9 per cent of these cases the cytologic report was negative for carcinoma in 2 per cent cell atypias were noted and in 13 8 per cent the cytologic picture was suspicious for carcinoma In 75 2 per cent of the 851 cases the diagnosis of mammary carcinoma was stated at aspiration biopsy

Cytological diagnosis of mammary cancer is considered *to be fully reliable and* cases judged to be operable are therefore referred to radical mastectomy without prior surgical biopsy

P S Persson T Ekman & K Hedberg NEEDLE BIOPSY OF THE PROSTATE A COMPARISON BETWEEN CYTOLOGICAL AND HISTOLOGICAL EXAMINATION

B Sandstedt HISTOLOGICAL AND CYTOLOGICAL FINDINGS IN BOWEN'S DISEASE OF THE VULVA

The cytological and histopathological findings in a case of Bowen's disease on the labium majus is described Repeated routine vaginal smears showed *advanced* dyskaryosis of superficial squamous cells but no definite cancer cells The origin of these cells was well demonstrated in histological sections which showed abundant

exfoliation of dyskaryotic superficial cells The vagina and the cervix were normal and vaginal smears taken after total excision of the vulvar lesions were negative

M Nasiell CYTOLOGICAL AND HISTOLOGICAL FINDINGS IN EARLY CARCINOMA IN THE BRONCHI AND THE UTERINE CERVIX

The cytology and histology of noninvasive and invasive cervical carcinoma is well known and it is possible to differentiate these lesions cytologically

Bronchial carcinoma is probably preceeded by intraepithelial changes but very little is known about such lesions 26 early and occult bronchial carcinomas have been reported The majority were detected by sputum cytology which revealed well differentiated squamous carcinoma cells a finding almost inevitably indicating invasive cancer

17 of the 26 reported cases and one case of our own (67 per cent) revealed early invasion in the sections while in intraepithelial cervical carcinoma early invasion occurs in only 5 18 per cent of the cases

3 other of our cases without clinical signs of malignancy but with differentiated squamous carcinoma cells in sputum and bronchial biopsies revealing carcinoma *in situ* with early invasion developed advanced bronchial cancer after 15–17 months

The type of diagnostic cell in most of the 27 cases and the fact that early invasion was found in 67 per cent indicates that carcinoma *in situ* and related lesions in the bronchial mucosa are more advanced lesions than carcinoma *in situ* in the uterine cervix and implays a poorer prognosis

E Johannisson P Kolstad & G Soderberg COMPARATIVE STUDIES ON THE CYTOLOGICAL VASCULAR AND HISTOLOGICAL PATTERN IN CARCINOMA IN SITU

Acta path et microbiol scandinav 66, 278-287, 1966

TRANSACTIONS OF THE MEDICAL MICROBIOLOGY DIVISION OF THE SWEDISH MEDICAL SOCIETY

Annual Meeting November 25-27, 1965

Received 19 1 66

Immunology

G Holm & P Perlmann The Wenner Gren Institute and Seraphimer Hospital, Stockholm IN VITRO CYTOTOXIC EFFECT OF LYMPHOID CELLS FROM RATS WITH EXPERIMENTAL AUTOIMMUNE NEPHROSIS

To be published in Clinical Experimental Immunology 1966

G Holm & P Perlmann The Wenner Gren Institute Stockholm CYTOTOXIC EFFECTS OF LYMPHOCYTES FROM UNSENSITIZED ANIMALS ON FOREIGN CELLS IN TISSUE CULTURE

In the presence of phytohaemagglutinin (PHA) lymphocytes from blood thoracic duct lymph nodes or spleen of unsensitized human subjects or animals aggregated to cells in tissue culture (= target cells) and damaged them within 48 hours The reaction required living lymphocytes and close contact between lymphocytes and target cells (Nature 203 841, 1964) No cell damage was observed in syngeneic combinations of lymphocytes and target cells In comparison with peripheral lymphocytes thymocytes showed no or only weak cytotoxicity on histoincompatible target cells (Nature 207 818, 1965) The lymphocytes seemed to remain intact during this reaction Neither could they be replaced by tissue cells (fibroblasts kidney cells etc) foreign to the cells used as targets Experiments where the cytotoxicity of lymphocytes was compared with their aggregation and transformation at different concentrations of PHA, indicated that target cell damage was more closely correlated to transformation of lymphocytes than to aggregation In the presence of polyvinyl pyrrolidon, an agglutinating agent without lymphocyte stimulating properties lymphocytes caused slight target cell damage An extract from Staphylococcus aureus which stimulated but did not agglutinate also induced a weak cytotoxic effect In the presence of both of these agents the cell damage was pronounced Therefore it seems that the cytotoxic capacity of immunologically competent cells from unsensitized animals on allogeneic or xenogeneic target cells is correlated both to aggregation and to their ability to respond to certain stimuli with growth and transformation

S E Seehag Institute for Virus Research Karolinska Institutet Stockholm MEMBRANE CULTURES OF LYMPHOID CELLS OF SPLEEN ORIGIN A SYSTEM FOR STUDIES OF ANTIBODY FORMATION IN VITRO

Lymphoid cells from rabbit spleen isolated by fractionation of spleen cells in Dextran were cultivated on membrane filters (2 5 × 10⁶ cells/culture) on a gas liquid interphase and humidified with 6 per cent CO_2 in air Restimulation of the

lymphoid cells *in vitro* with purified polio virus antigen evoked secondary antibody responses of up to 40 days duration The molecular type of antibody produced in the secondary responses as well as their kinetics depended upon the number of com petent cells committed to 19S and 7S antibody synthesis present in the cultures and on the antigen cell ratio used when restimulating *in vitro*

Induction of secondary *in vitro* antibody responses did not seem to require the presence of macrophages in the present system This conclusion is based on the following observations *in vitro* antibody responses could be effectively inhibited by the addition of macrophages to the lymphoid cell cultures prior to their stimula tion with antigen the same effect was observed when antigen was incubated with macrophages which subsequently were added to the lymphoid cells

Erna Moller Dept of Tumor Biology Karolinska Institutet Stockholm CONTACT INDUCED CYTOTOXICITY MEDIATED BY HISTOINCOMPATIBLE CELLS

Non immune allogeneic lymphoid cells are cytotoxic to normal and neoplastic target cells in tissue culture provided PHA or heterologous antiserum are also present These agents probably act by causing aggregation between the two cell types Syngeneic cells have no effect under identical experimental conditions

Immune lymphoid cells are cytotoxic without the addition of aggregating agents It seemed possible that non immune lymphoid cells were stimulated to perform a primary immune response *in vitro* leading to target cell death However it was found that semi syngeneic lymphoid cells from an F_1 hybrid mouse admixed to target cells from one of the parental genotypes were as active as allogeneic cells to cause cytotoxicity Since F_1 hybrid cells are genetically incompetent to react immu nologically against the parental genotypes it is unlikely that the cytotoxic reactions were mediated by conventional immune reactions Furthermore pretreatment of the lymphoid cells with 1500–3000 r did not abolish the cytotoxic activity of these cells

Preliminary experiments showed that also neoplastic cells of lymphoid and fibro blastic origin are competent to mediate cytotoxicity reactions both *in vitro* and *in vivo* if they are aggregated to allogeneic target cells It seems most likely there fore that cytotoxicity is caused by close contact between histoincompatible cells The actual mechanism of target cell death is not known as yet

The present findings are in agreement with the results of Hellstrom concerning the "syngeneic preference or allogeneic inhibition phenomenon

Part of these data has been published in Science (*147* 873 1965) and in Nature (*208* 2601 1965)

S Britton Dept of Tumor Biology Karolinska Institutet Stockholm
IMMUNOLOGICAL REACTIONS AGAINST A LIPOPOLYSACCHARIDE ANTIGEN STUDIED AT THE CELLULAR LEVEL

Immunological reactions against the somatic O antigen of the gramnegative bacteria E coli were studied at the cellular level by the agar plaque technique described by Jerne Inbred mice were immunized with a bacterial vaccine and were subsequently sacrificed at regular intervals and their spleens tested for number of plaqueforming cells (PFC) against sheep red cells sensitized with the coliantigen The number of PFC increased exponentially and reached a maximum after five days whereafter they decreased to background levels after 10 days 12 15 days after immunization a new

peak appeared with approximately the same value number of PFC. Throughout the subsequent 50 day testperiod the number of PFC showed regular cyclical fluctuations Since a primary immunization with this antigen stimulates only 19S antibody production and since the antigen is known to remain biologically active for a long time, as revealed by its ability to stimulate new antibody production, it appears likely that the cyclical antibody production may be explained by a feed back inhibition Five days after the immunization the antigenic determinants would be blocked by the synthesized 19S antibodies This would inhibit further stimulation to antibody production However, since the 19S antibodies are very shortlived they would rapidly disappear and the stable coli antigen would become free to stimulate a new production of antibodies and this process would be repeated

In another experimental system lymphoid and bone marrow cells were incubated *in vitro* with the lipopolysaccharide antigen of E coli The cells were then washed and injected into syngeneic mice, which previously had been irradiated with a lethal dose of x irradiation (900 r) Three weeks later the animals were injected with the bacterial vaccine of E coli The recipients of cells treated with the coli lipopolysaccharide did not respond with cellular antibody synthesis whereas control animals injected with cells treated with a non cross reacting lipopolysaccharide showed a normal cellular immune response to the E coli antigen Thus the treatment *in vitro* of immunologically competent cells with a lipopolysaccharide antigen resulted in sustained and specific immunological unresponsiveness whereas the same dose of antigen injected into the control animals constantly resulted in antibody synthesis

Bo Fjällbrant Department of Bacteriology, University of Göteborg SEROLOGICAL STUDIES OF SEMINAL ANTIGENS

Rabbits were injected with semen washed seminal spermatozoa and seminal plasma from one fertile donor and with spermatocele spermatozoa The injections were given once a week for four weeks the first time both subcutaneously in Freund's adjuvant and intravenously and subsequently only intravenously The immune sera were studied with a modified Ouchterlony diffusion in gel technique immunoelectrophoresis Kibrick's gelatin agglutination test and a method for determining the spermimmobilizing effect The preparations which contained spermatozoa were freezepressed in order to crush the spermatozoa and then frozen and thawed ten times

The following results were obtained:

The anti spermatocele spermatozoa serum gave no precipitation lines with semen but had a slight agglutinating and immobilizing effect on intact spermatozoa

Semen and seminal plasma each formed one line with the anti seminal spermatozoa serum These lines formed a reaction of fusion No line was developed when blood plasma was tested with anti seminal spermatozoa serum The antiserum had a moderate agglutinin titre but the immobilizing effect was slight

The anti semen serum developed 16 lines with seminal plasma and showed a high agglutinin titre and a high immobilizing effect With human blood plasma the anti semen gave 6 lines Seminal plasma gave with an anti human blood plasma serum at least 9 lines Three of the lines in this spectrum were identified as albumin transferrin and gammaglobulin precipitate by comparative immunoelectrophoretic analysis

B. Björklund, J. E. Paulsson & B. Noren, Cancer Immunology Section, National Bacteriological Lab., Stockholm, Sweden: ANTI HELA GLOBULIN REACTIONS WITH ANTIGENS FROM POOLED NORMAL AND MALIGNANT HUMAN TISSUES

Horse anti HeLa serum was used to indicate cross reacting antigens in a pool of 65 individual human carcinomas as compared to a pool of 248 normal tissues from 52 individuals. Two non humoral tumour antigens could be demonstrated by immunoprecipitation in agar gel and tanned cell haemagglutination technique. These reactions were inhibited by pretreatment of the anti HeLa serum by the tumour pool but not by the normal one. Horse anti normal tissue serum did not give rise to reactions with these antigens but reacted with normal antigens.

One of the two tumour pool antigens was DNA protein, the other being of polysaccharide nature.

The specific antibodies to the DNA protein antigen could be separated from the antibodies to the polysaccharide by means of dialysis against 0.02 M NH_4HCO_3 buffer pH 8.5. The resulting precipitate and supernatant were each separately subjected to molecular sieve filtration on Sephadex G–200.

The IgG (7 S) from the precipitate contained antibodies which gave rise to

1. A gel precipitate line with the polysaccharide antigen
2. Cytotoxicity in monolayer cultures of He La-cells
3. No agglutination of tanned cells labelled with extracts of tumour or normal pool

The IgG (7 S) from the supernatant caused

1. No cytotoxicity
2. A gel precipitate line with the DNA protein
3. Agglutination of tanned cells labelled with the purified DNA protein

Following identical separation procedures the tumour extract yielded two active peaks while the normal extract contained virtually nothing to compare with. The purification included extraction, isoelectric precipitation, stabilization, electrophoresis and column chromatography.

R. Lagercrantz, S. Hammarström, J. Perlmann & B. E. Gustafsson, The Wenner Gren Institute for Experimental Biology, University of Stockholm, the Department of Pediatrics, Karolinska Hospital and the Department of Germfree Research, Karolinska Institute, Stockholm: COLON ANTIBODIES IN HUMAN ULCERATIVE COLITIS AND THEIR RELATIONSHIP TO BLOOD-GROUP AND BACTERIAL ANTIGENS

Phenol water extract of sterile rat-colon contains an antigen identical or similar to human colon antigen. Patients with ulcerative colitis have significantly higher antibody titres against this antigen than healthy controls and patients with acute or chronic diseases of the gastro intestinal tract (Salmonellosis, bacillary and amoebic dysenteria, colon cancer, chronic diarrhoea of unknown aetiology and malabsorption syndromes). The rat antigen contains blood group substances A and H. Most of the background antibodies in sera from controls could be absorbed with A erythrocytes. The colon antigen is a mucopolysaccharide cross reacting with antigen(s) from *Staphylococcus aureus* and *Escherichia Coli* O 14 but not with 7

other O types of E. Coli or *Streptococcus haemolyticus* Pneumococci or Enterococci. In indirect immunofluorescence sera from patients with ulcerative colitis stained goblet cells and surface mucous of sterile rat colon specifically This reaction could be blocked by prior absorbtion with the rat colon antigen A significant correlation was demonstrated between indirect haemagglutination and indirect immunofluorescence

Females with ulcerative colitis have significantly higher antibody titres than males No correlation was found between titres and duration severity distribution of colon lesions or the presence of extra-colonic manifestations Patients operated on with pancoloproctectomy many years earlier had titres similar to those with *active disease Relatives to patients with ulcerative colitis often have elevated anti* body titres as compared with controls (preliminary results) Some individuals might have a genetically determined tendency to develop auto antibodies and/or disease on stimulation from the above mentioned cross reacting antigens in bacteria common in the gastrointestinal tract

Virology

F Norrby Department of Virus Research Karolinska Institutet Stockholm THE HAEMAGGLUTININ OF ADENOVIRUS TYPE 3

To be published in Virology 1966

Gunnar Svart-Malmberg Section of Virology Department of Clinical Bacteriology Karolinska sjukhuset Stockholm STUDIES OF THE EARLY ANTIBODY RESPONSE TO ADENOVIRUS TYPE 7

A rise of thermolabile as well as mercaptoethanol sensitive neutralizing activity was demonstrated in rabbits and guinea pigs during the first days after a primary *injection with adenovirus type 7 antigen*

After preparative ultracentrifugation in sucrose gradient the neutralizing capacity was found in the fractions from which macroglobulins are usually recovered for a longer period after immunization than it would appear from the observations with heat and mercaptoethanol treatment

Since mercaptotehanol was shown to destroy the haemolytic function of complement both this compound and heat inactivation may interfere with a complement *dependent antibody function However the addition of guinea pig* complement did not restore the neutralizing capacity of animal sera treated with heat or with mercaptoethanol Possibly both early antibody and complement components necessary for neutralization are both heat and mercaptoethanol sensitive

Wiederman et al (Proc Soc Exptl Biol Med *115* 609 1963) have reported that the complement fixing ability of 7S antibodies is destroyed by mercaptoethanol In contrast the complement fixing titres of some human sera tested against adeno and herpes simplex virus antigens remain practically unaffected after mercaptoethanol treatment In some other presumably rather late convalescent sera however considerable decrease was observed after treatment

B Hoorn Institute of Medical Microbiology University of Lund, Sweden and The M R C Common Cold Research Unit Salisbury England A NEW RHINOVIRUS ONLY DECTABLE BY ORGAN CULTURE TECHNIQUE

The results are presented in two papers A new virus cultivated only in organ cultures ciliated epithelium and Inoculation of a novel type of rhinovirus (H S) to *human volunteers*

To be published in Archiv fur die gesamte Virusforschung 1966

J A Espmark & A Fagraeus National Bacteriological Laboratory Stockholm DEMONSTRATION OF RABIES ANTIBODIES BY MIXED HAEMADSORPTION

The mixed haemadsorption (M H) technique (Nature *190* 370 1961 Immunology 9 161 1965) earlier used for the demonstration of antibodies to vaccinia canine distemper and RS viruses was applied to rabies virus infected cell monolayers of HEp 2 and green monkey kidney (GMK) cells Using sera known to contain antibody to rabies virus but no antibodies to uninfected cells adsorption of antigamma globulin-coated erythrocytes was obtained in cultures heavily infected with the HDCS adapted Flury HEP rabies strain of *Wiktor Fernandes & Koprowski* (Over 50 per cent infected cells as checked by direct immunofluorescence were required) The positive M H suggested the presence of surface located viral antigen on infected cells

For antibody assay serial dilutions of testsera were placed by filter paper discs upon a *thin agar layer covering the infected cell sheet in flasks Following a dif* fusi n time of 3 days at room temperature agar and discs were removed and the indicator erythrocyte suspension added Positive sera gave round haemadsorption zones the diameter of which was linearly related to the log serum dilution A number of monkey antirabies sera (kindly supplied by dr *T Wiktor* The Wistar Institute) were assayed in parallel by mixed haemadsorption and by neutralization tests intracerebrally in suckling mice Antibody titres obtained by the two test were of the same order of magnitude indicating similarity with respect to sensitivity

B Bloth & F Norrby Department of Virus Research Karolinska Institutet Stockholm FRACTIONATION OF RS VIRUS MATERIAL IN CsCl EQUILIBRIUM DENSITY GRADIENTS

Concentrated virus material has been fractionated by equilibrium density ultra centrifugation in CsCl gradients After centrifugation of a mixture with a starting density of 1.22 g cm^3 the infectivity peak was found to accumulate at this particular density The major part of the complement fixing antigens was found in fractions with a density of more than 1.25 g cm^3 but activity was also associated with frac tions with lower density Treatment with Tween 80 and ether eliminated all infec t vity Centrifugation of Tween ether treated material in a gradient with a starting density of 1 28 g cm^3 showed a spread of CF antigens over a density range from 1.25 to 1 33 g cm^3 Virusmaterial has also been fractioned by zonal rate centrifuga tion in gradients of CsCl solutions Two populations of particles were found One sedimented rapidly and contained all infectivity and some CF activity The virus material in this fraction had a density less than 1.27 g cm^3 The slowly sedimenting virus products (soluble antigens) showed only CF activity When these soluble antigens were rerun in a CsCl equilibrium density gradient they accumulated at

densities varying between 1.25 and 1.33 g cm³. Tween ether treatment resulted in almost no changes in the distribution of the CF activity of non infectious CF antigens

Bacteriology

Gunnel Biberfeld The National Bacteriological Laboratory, Stockholm ANTIBODIES IN PATIENTS WITH MYCOPLASMA PNEUMONIAE INFECTION

Absorption of positive convalescent phase sera with Mycoplasma pneumoniae antigen removed complement fixing (CF) indirect haemagglutinating (IHA) and fluorescent antibodies without reducing the titre of the cold agglutinins. These agglutinins could be absorbed with human erythrocytes without a change in titre of the specific antibodies to M. pneumoniae and human erythrocytes. After ultracentrifugation of convalescent sera in a sucrose gradient cold agglutinins were found in the 19S fraction while specific antibodies to M. pneumoniae were demonstrated in the 7S as well as in the 19S fraction. In several sera taken one month after the onset of illness the IHA antibodies were predominantly of 19S type while the CF antibodies were predominantly of 19S type while the CF antibodies were mainly of 7S type. In one case the IHA antibodies were both 19S and 7S after one month while 6 months later almost all IHA antibodies were 7S. Another case however had 19S IHA antibodies exclusively even after 7 months.

A fourfold rise of CF antibodies to mouse lung antigens was found in 13 of 53 cases (25 per cent) of serologically diagnosed mycoplasma pneumonia.

A. Frisk The Central Bacteriological Laboratory of Stockholm City, Stockholm SEROLOGICAL STUDIES ON ASPERGILLUS FUMIGATUS IN PATIENTS WITH VERIFIED PULMONARY ASPERGILLOSIS AND OTHER RESPIRATORY DISEASES

The immunological response in man against low pathogenic fungi is discussed. The high frequency of cross reactions depends upon related antigen fractions in the mycelial wall or in the spores. In most cases the antigens have been prepared with rough methods (treatment with acid, alkali or ethanol etc.) resulting in denaturation of the proteins. The author has prepared a precipitating antigen from Asperg. fum. which is harvested after 5 days growth in a low molecular substrate at pH 6.5 with continous aeration. The mycelial mass is centrifuged at 4° C and afterwards washed several times. A suspension of the mycelium is made in phosphate buffer pH 7.0 and frozen to −30° C. After desintegrating the cells in the X-press ad modum Edebo the mass is extracted in phosphate buffer and afterwards concentrated in "Cellugel" ad modum Palmstierna. The concentrate contains cell proteins, nucleic acids and high molecular weight carbohydrates which are not so hard fixed to the cell wall. Gel filtration was subsequently performed on Sephadex G 100 at +4° C and the antigen activity was eluted in the void volume.

Sera from clinical pulmonary aspergillosis (7 cases verified by histological examination in 5/7) gave sharp linear precipitates with gel diffusion technique in all 7 cases. 99 cases with other pulmonary diseases gave precipitates in 12 (among those 2 cases of eosinophilic pulmonary infiltrates, 1 case of thresher's lung and 5 cases of pulmonary mycosis). A more detailed report of the investigation will be published in Scand. J. Resp. Dis. 1966.

S Winblad & N H Sternby Institutes of Clinical Bacteriology and Pathology, (University of Lund), General Hospital Malmo PASTEURELLOSIS X—A HUMAN ENTERIC INFECTION WITH ACUTE TERMINAL ILEITIS OR MESENTERIC LYMPHADENITIS

The O antigen of a strain of Pasteurella X (Yersinia enterocolitica) has been used in an agglutination reaction with the following results High titres of agglutinins with typical antibody curves during 4 6 months occurred in 70 per cent of the cases of acute terminal ileitis and 50 per cent of cases of mesenteric lymphadenitis All of these patients had been operated upon on the indication of a presumed appendicitis

In healthy persons and patients with various internal diseases elevated agglutinin titres were found in 3 5 per cent of 1 231 persons In this group there was a con nection between high agglutinin titres and histories of diarrhoea or abdominal pain About 25 per cent of patients with acute appendicitis showed elevated agglutinin titres during convalescence Among 100 non selected cases of diarrhoea 5 per cent had elevated agglutinin titres Among patients with Crohn s disease, only a few showed moderately elevated titres

In three cases of acute terminal ileitis or mesenteric lyphadenitis Pasteurella X was isolated from the appendix or mesenterial lymph nodes

In several cases the histological examination revealed the presence of leucocyte containing micro abscesses in the germinal centres of the lymphatic tissue This may be a characteristic histo pathological lesion in Pasteurellosis X

In contrast to Masshoff's disease where high agglutinin titres against Pasteurella pseudotuberculosis occeur already at the time of the onset of acute symptoms the agglutinins against Pasteurella X were regularly demonstrable only after a lapse of about a week

Throughout this study the sera have been tested also for agglutinins against pseudotuberculosis In this way two cases of Masshoff s disease have been diagnosed

Pasteurellosis X is an enteric infection which is not uncommon in Sweden Geographic and temporal concentrations of the cases have not been of a degree which might indicate an epidemic spread No family infections have been noted

The serological diagnosis of the disease with the demonstration of O agglutinins is distinct No cross reactions with antigens from other Pasteurellae of Entero bacteria have been noted

S Holm D Braun & Jane Jonsson Department of Bacteriology University of Gothenburg INVESTIGATION CONCERNING A HYPERTENSIVE FACTOR FROM TYPE 12 STREPTOCOCCI

We have earlier shown that intravenous injection of intracellular material from type 12 streptococci isolated from a patient with acute glomerulonephritis could produce nephritis in rabbits which histopathalogically was similar to the post streptococcal glomerulonephritis in human subjects Clinical signs of nephritis with haematuria albuminuria and hypertension were also noted The hypertension as measured by the ear capsule method was initiated within a few minutes after the injection and lasted for several weeks

Purification of this hypertensive factor has now been performed using Sephadex G–25 The elution volume for this factor corresponded to 4 times of the void volume of the column Ultraviolet measurements showed that the factor had its absorption maximum at 260 mμ Infra red spectrophotometry pointed to a cyclic compound

By injection of this substance into A renalis the blood pressure increased 3 5

times of the initial value with 2 minutes as measured by the ear capsule method and remained high during the next two weeks. A temporary decrease of the high blood pressure was noted if α methyl Dopa was injected within some minutes after introduction of the hypertensive factor. No difference in the hypertensive effect of the substance could be noted if injections were made into A. renalis, V. portae or aorta. Whether this substance is responsible for the nephrotoxic activity of intra cellular material from type 12 streptococci cannot be stated at the moment. Investigations concerning this problem are in progress.

P. Branefors Department of Bacteriology, University of Gothenburg, Gothenburg.
SOLUBLE ANTIGENS OF HAEMOPHILUS INFLUENZAE STUDIED BY MEANS OF DIFFUSION IN GEL TECHNIQUES

Encapsulated Haemophilus influenzae of the capsular types a–f and an R variant of the type d strain have been investigated for antigenic composition by means of the double diffusion in gel technique and by immunoelectrophoresis. Antigens for the immunodiffusion (ID) analyses consisted of washings from young cultures on Levinthal agar plates. Antisera were prepared in rabbits against suspensions of whole bacteria.

The ID patterns of the washings from encapsulated strains tested with their homologous antisera consisted of two precipitation lines. For each antigen one of these lines represented the type-specific capsular substance. The type b strain contained in addition to capsular substance b a small amount of capsular substance a. The antigen representing the second line was found also in the R variant of the type d strain. The two type a strains investigated possessed at least two capsular antigenic components, one in common and the other strain specific.

The two antigens appeared within the void volume when filtered through Sephadex G 200. They could, however, be separated on DEAE-cellulose with gradient elution.

A. Lindberg, H. Bucht & I. O. Kallings The National Bacteriological Laboratory, Stockholm and the Renal Clinic, St. Eriks Hospital, Stockholm.
TREATMENT OF CHRONIC PYELONEPHRITIS WITH GENTAMICIN

Gentamicin is a new antibiotic related to the streptomycin-kanamycin group with bactericidal activity against Gram negative bacilli and staphylococci. When tested against 229 freshly isolated Gram negative rods the mean MIC (inhibitory) value was 1.4 mcg/ml, the mean MBC (bactericidal) value 3.7 mcg/ml. An alkaline pH enhanced the activity of gentamicin 15 to 30 fold when the pH was increased from 6 to 8.

Gentamicin therapy was given intramuscularly for 7 days twice daily to 12 patients with urinary tract infections (all severe chronic cases) and to one patient with Gram negative septicaemia. All patients were treated with reduced doses adjusted to the renal function, i.e. a total daily dose of 0.4 to 1.1 mg/kg. Peak levels in serum 2–6 mcg/ml were achieved after 1–4 hours. The drug showed no tendency to accumulate in the serum with the doses used. In the urine high peak levels 14–59 mcg/ml appeared within 4 hours after injection. After 12 hours the urine levels were still bactericidal for most of the Gram negative strains tested. During therapy 12 out of 13 were cured; after 6 months 5 out of 7 had relapsed. The patient with septicaemia continuously requiring haemodialysis was dramatically cured. No vestibular damage was encountered in this series but has been reported by others.

A Wallerstrom Department of Bacteriology Lund University, Lund PRODUCTION OF ANTIBIOTICA OF EPIDERMOPHYTON FLOCCOSUM

To be published elsewhere

Ingrid Grabell & B Zetterberg National Bacteriological Laboratory, Stockholm, Sweden ANTHRAX IN TEXTILE WORKER

A fatal case of anthracic sepsis occurred in a textile worker at Kinna in the autumn of 1964 The deceased was a 53 year old man who had worked in the same mill for several years He fell ill with catarrhal symptoms After 3 days his condition rapidly deteriorated and he exhibited great anxiety Later the same day he became unconscious and died a few hours later

Bacillus anthracic was found in samples from cerebrospinal fluid and blood The cerebrospinal fluid was mixed with blood No postmortem was made The symptoms agree with those reported in the literature for inhalation anthrax

In conjunction with this case samples were collected from several batches of wool at the mill for bacteriological examination Bacillus anthracis was found in several samples including the batch used prior to the man s illness

The epidemiology of the disease is described and preventive measures are discussed

This is the first case in a textile worker reported in Sweden since the reporting of anthracic bacterial infection became compulsory in 1919

S Wittboldt INTACT AN ADB SYSTEM FOR HOSPITAL HYGIENE AND EPIDEMIOLOGY

F Ernerfeldt I O Kallings O Ringert & L Silverstolpe The National Bacteriological Laboratory Stockholm Sweden BACTERIOLOGICAL HAZARDS IN PHARMACEUTICAL MANUFACTURING

In 1963 pharmaceutical tablets were found to be heavily contaminated with bacteria (E coli) and in 1964 an eye ointment causing severe eyeinfections was found to contain Pseudomonas pyocyanea

These two incidents induced an investigation of the presence of bacterial contaminants in pharmaceutical preparations without official requirements of sterility Samples from 460 different batches were studied and from 203 of these (44 per cent) bacteria were isolated B subtilis Staph albus and yeast and mould fungi were most frequent but in several cases Staph aureus E coli K aerobacter and Ps pyocyanea were isolated More than 65 per cent of the ointments and powders studied were found to be contaminated in some cases by more than 10^4 bacteria per gram Contaminations were also frequent in pills tablets and eye ointments

In factories in which production arrangements were of a good hygienic standard the rate of contamination products was found to be low

In some cases the contaminating bacteria derived from the raw materials used (e g potate starch) while in other cases they were introduced or allowed to multiply during the production where the growth facilities sometimes were quite favourable

Increased hygienic standard in manufacturing bacteriological production control and bacteriological standards or requirement of sterility for some products are suggested

interesting difference was disclosed when an alkaline phosphatase reaction was applied. The mesenterial mast cells showed a moderate phosphatase activity but no such activity could be demonstrated in the mucosal mast cells. The reason for this difference was a dissolution of the granules of the mucosal mast cells during incubation in the substrate. Even incubation in distilled water at 37° C destroyed the granules.

Against this background it is interesting to note that more recent papers often deny the existence of mast cells in intestinal wall. *Mota, Ferri & Yoneda* (1956), for example, studied the distribution of mast cells in the digestive tract of laboratory animals. Using preparations fixed in alcoholic lead acetate—acetic acid, sectioned frozen and stained with toluidine blue, they found that mast cells could be demonstrated only in tongue, oesophagus and forestomach and were virtually non-existent in the rest of the intestinal wall. *Riley* (1959) reported much the same observations from tissues treated in different ways, including fixation in 4 per cent basic lead acetate solution, paraffin embedding and staining with toluidine blue. Conversely, *Lindholm* (1960), using a similar histological procedure, could demonstrate what he considered to be ordinary mast cells in the submucous coat of rat intestine. *Räsänen* (1960, 1961, 1962), using the same technique, studied the effect of ACTH and steroids on mast cells in the rat gastric wall. His findings indicate that mast cells are quite numerous in the superficial part of the gastric mucosa.

It seems clear from the foregoing that the intestinal mucosa of the rat contains mast cells. These cells are often overlooked, presumably due to technical circumstances. The fixation is certainly the most sensitive point in the technique for demonstration of mast cells. Therefore the effect of fixatives on intestinal mast cells was investigated. It will be demonstrated in the following that the gastrointestinal mucosa of the rat contains a great number of cells having metachromatic granules which do differ in many respects from mast cells elsewhere. A new fixation technique will be described which gives good preservation of both these types of cell.

MATERIALS AND METHODS

The investigation was performed on altogether 20 male and 4 female albino rats of the Sprague Dawley strain (Anticimex AB, Stockholm) as well as 2 male and 2 female Wistar rats of a strain bred at this laboratory. The rats were fed [illegible]mmercial pellets and allowed to eat and drink *ad libitum*. They were killed by [illegible] ation between 12 noon and 4 p.m. without prior starvation. The back skin was [illegible] with *an electrical clipper*. Suitable tissue samples were dissected out as quickly as [illegible] ble and fixed. The general histological procedure comprised the following standardized [illegible] r cent ethanol [illegible] ver night wh[illegible] [illegible] n and preparation of 5 μ sec[illegible] nada [illegible] al am[illegible]

The eff[illegible] as studied [illegible] n 12 male Sprague D[illegible] ups of 4 rats each. Pieces of dorsal skin and intestine from the same rat were paired. The t[illegible] ues from

one such pair from each rat were fixed together in each of the fixatives tested, and subjected to the same histological procedure They were stained on the same slide, in order to minimize random variations in staining and dehydration The preservative efficiencies were estimated and arbitrarily quantified. The term preservation will only be used to connote preservation of stainability with the adopted staining technique

In another group of rats the distribution and morphology of mast cells were studied The rats were 8 Sprague-Dawley males weighing 154–167 g, 4 Sprague-Dawley females weighing 163–174 g, plus two male and two female Wistar rats weighing 144 168 g From the male Sprague Dawley rats pieces were excised from the following organs and immediately fixed in either 4 per cent formaldehyde (unbuffered) or in an isotonic formaldehyde-acetic acid mixture, IFAA (see below)· oesophagus (transverse section from distal portion, about 0 5 cm above cardia), stomach (longitudinal section along minor curvature from pylorus to cardia), duodenum (transverse section about 0 5 cm below pylorus), jejunum, ileum and colon transversum (transverse sections from central portions) Furthermore pieces of tongue, trachea, bronchus, lung, thymus, axillary lymph nodes, spleen, liver, pancreas, dorsal skin and ears were taken A similar selection of pieces from the digestive tract were taken from each of the Sprague-Dawley females as well as from the male and female Wistar rats From female rats specimens were also taken from uterus and fallopian tubes All tissues from the latter 8 rats were fixed in IFAA only

Fixatives

[illegible]

per cent formaldehyde with Mc Ilvaine's citric acid disodium phosphate buffer at [illegible]

Alcoholic lead nitrate (*Lillie* 1954) lead nitrate 8 g 35 per cent formaldehyde 10 ml distilled water 10 ml, ethanol 80 ml

Basic lead acetate acetic acid ethanol (*Mota et al* 1956) basic lead acetate 1 g [illegible]

Bouin s fixative (*Romeis* 1948) saturated aqueous solution of picric acid 15 ml, 35 per cent formaldehyde 5 ml acetic acid 1 ml

Susa according to Heidenhain (*Romeis* 1948) mercuric chloride 4 5 g sodium chloride 0.5 g distilled water 80 ml trichloroacetic acid 2 g acetic acid 4 ml 35 [illegible] ous solution of [illegible] ml, acetic acid 10 ml

Zenker s fluid (*Romeis* 1948) potassium dichromate 2 5 g sodium sulphate 1 g distilled water 100 ml mercuric chloride 5 g acetic acid 5 ml

Staining Procedure

The sections were stained in an 0.5 per cent aqueous solution of toluidine blue (Toluidinblau nach Hover Merck AG Darmstadt (I No 52040 2nd Ed.) at pH 4 (Mc Ilvaine s citric acid-disodiumphosphate buffer) The standard staining time was 45 seconds To avoid overlooking weakly staining mast cells all sections in experiment I (see below) were in additi n stained for 3 minutes. This grossly overstained

most sections Material fixed in Bouin Susa Subtrie and Zenker showed however lower affinity for toluidine blue manifested by very pale staining These sections were in addition stained for 10 minutes without appreciable increase in basophilia The dehydration technique was therefore modified for these sections (see below)

Immediately after staining the sections were rinsed rapidly in distilled water (15 seconds) and then passed rapidly through 70 per cent 96 per cent and absolute ethanol (15 seconds in each) to xylene Tissues fixed in Bouin Susa Subtrie and Zenker were in addition dehydrated in the following manner (i) A 15 second rinse in alkaline distilled water (a few drops of 2N NaOH to approximately pH 8) followed by alcohol dehydration as above (ii) a rinse in alkaline distilled water followed by dehydration in tertiary butanol (*Levine* 1939) and (iii) a rinse in ordinary distilled water followed by dehydration in tertiary butanol Each of these procedures increased the orthochromatic background color, and the overall affinity of the tissues for toluidine blue became roughly equal to that of sections fixed in 4 per cent formaldehyde and stained for 3 minutes

RESULTS

1 *Preservation of Intestinal and Dermal Mast Cells by Different Fixatives*

All the fixatives tested (Table 1) had wholly compatible effects on each of the four rats

The number of dermal mast cells was approximately the same however the tissues had been fixed Staining for 45 seconds generally imparted an intense dark violet colour to the mast cells Nuclei could seldom be distinguished owing to the dense and heavily stained granulation The cell margins were as a rule distinct Preparations fixed in Subtrie constituted an interesting exception in that their mast cells displayed a cherry red colour and had cytoplasmic granules appearing as discrete dark red specks against an apparently homogeneously red background The nuclei appeared very distinct The cell margins were mostly sharp Overstaining by prolonging the staining time to 3 minutes did not remove the reddish metachromasia of the mast cells it merely turned darker The most notable exception however, was presented by the mast cells of the alcohol fixed preparations Like those just mentioned these mast cells showed a reddish metachromasia but in addition their outlines were diffuse and surrounded by metachromatic haloes This appearence of the mast cells was most marked in the preparations fixed in the higher concentrations of ethanol The mast cells in preparations fixed in 50 per cent ethanol were better preserved and had only slightly blurred margins

---

Figs 1 4

Fig 1 Skin Large mast cells with heavily stained granules

Fig 2 Gastric mucosa Smaller mast cells apparently interposed between epithelial cells

Figs 3 and 4 Intestinal mucosa Small mast cells less densely granulated than those of skin The cells are situated within the lamina propria

All sections were fixed in IFAA and stained with 0 5 per cent toluidine blue at pH 4 Magnification 1200 ×

TABLE 1

Preservation of Intestinal and Dermal Mast Cells by Different Fixatives

Fixative	Intestinal mast cells	Dermal mast cells
Formaldehyde, 4%	(+)	++
Formaldehyde, 4% neutral buffered	0	++
Formaldehyde, 4% pH 3 8 buffered	(+)	++
Bouin	0	++
Susa	0	++
Subtrie	0	++
Carnoy	+	++
Zenker	0	++
Ethanol 96%	0	(+)
Ethanol 70%	0	(+)
Ethanol, 50%	0	++
Basic lead acetate 4%	+	++
Alcoholic lead nitrate	+	++
Basic lead acetate acetic acid ethanol	++	++
Sublimate ethanol	0	++
Cetyl pyridinium chloride-formaldehyde	(+)	++
Aminoacridine ethanol	0	++

In intestinal preparations, stained for 45 seconds, mast cells could be demonstrated following only a few fixation treatments The best results were achieved by fixation in the basic lead acetate-acetic acid -ethanol mixture according to *Mota et al* All sections so fixed exhibited a large number of mast cells with a sparse reddish granulation However, the presence of precipitates in some sections was disturbing The results were less good with 4 per cent basic lead acetate and Lillie's alcoholic lead nitrate The mast cells demonstrated after these fixations were similar in appearance to those in preparations fixed in Mota's solution but seemed conspicuously fewer and, at any rate in preparations treated with Lillie's alcoholic lead nitrate, more weakly stained Notably, however, treatment with 4 per cent basic lead acetate yielded sections with very poor tissue fixation, which interfered with assessment In addition the sections contained coarse precipitates Specimens fixed in Carnoy's solution exhibited mast cells with faintly red metachromatic granules No mast cells could be identified after 45 seconds' staining in preparations fixed in any of the other solutions After overstaining of the sections by means of a staining time of three minutes, occasional, faintly red staining mast cells could be demonstrated in intestinal preparations fixed in 4 per cent formaldehyde either unbuffered or acid, and in the cetylpyridinium chloride-formaldehyde mixture Overstaining did not improve the demonstrability of mast cells in the other preparations In those intestinal specimens in which mast cells could be demonstrated after the short staining time these become more intensely violet but also invariably more difficult to distinguish against the more intensely orthochromatic background basophilia

2 *Preservation of Intestinal and Dermal Mast Cells by Formaldehyde and Acetic Acid*

The concentrations of the solutions tested are specified in Table 2, which also shows the result of a rough assessment of the degree of mast cell preservation Observations made on the 4 rats were wholly compatible with one another

TABLE 2
Preservation of Intestinal and Dermal Mast Cells by Formaldehyde and Acetic Acid

Fixative		Intestinal mast cells	Dermal mast cells
Acetic acid	0 5% v/v	++	(+)
	1	++	(+)
	2	+	(+)
	4	(+)	(+)
	8	(+)	(+)
	16	(+)	(+)
	32	(+)	++
	64	0	++
	100	0	++
Formaldehyde	0 4% w/v	++	++
	0 8	(+)	++
	1 6	0	++
	3 2	0	++

As expected, low acetic acid concentrations turned out to produce a very poor tissue fixation, yielding loose and bloated preparations from which it was practically impossible to cut untorned sections Only 100 per cent acetic acid yielded acceptable tissue fixation

In intestinal preparations fixed in low acetic acid concentrations it was possible nevertheless to demonstrate sharply outlined mast cells with well preserved and intensely stained cytoplasmic granules The preservative effect of acetic acid solutions for intestinal mast cells diminished with increasing concentrations, and at concentrations from 32 per cent no mast cells whatsoever could be seen in the sections

The action of acetic acid on dermal mast cells was different After fixation in weak acetic acid solutions, the mast cells in the dermal preparations had a reddish tint the cytoplasm appeared diffusely stained and the darker violet granules appeared silhouetted against it Here and there the cell margins were indistinct and the cells surrounded by metachromatic haloes At higher concentrations of acetic acid, dermal mast cells had less blurred margins and were stained darker violet

Formaldehyde yielded much better tissue fixation than acetic acid The disadvantages of the formaldehyde fixation predominantly shrinkage and hardening of the tissues, made themselves felt more particularly on skin preparations, the sections of which often tended to crack

Strange to say, formaldehyde solutions with the very low concentration of 0.4 per cent resulted in satisfactory demonstration of intestinal mast cells. They occurred in great quantities and had granules presenting a reddish metachromasia. A mere doubling of the formaldehyde concentration resulted in a conspicuous change. The sections now contained definitely fewer and less brightly stained mast cells. After fixation at still higher concentrations the sections of intestine appeared completely devoid of mast cells.

Dermal mast cells could be demonstrated after fixation in formaldehyde solutions at all concentrations tested.

3 *Preservation of Intestinal and Dermal Mast Cells by Mixtures of Acetic Acid and Formaldehyde in Low Concentrations*

The composition of the solutions tested and the results they produced are found in Table 3. The observations made on the four rats were wholly compatible with one another.

TABLE 3

Preservation of Intestinal and Dermal Mast Cells by Mixtures of Acetic Acid and Formaldehyde in Low Concentrations

Fixative			
Formaldehyde % w/v	Acetic Acid % v/v	Intestinal mast cells	Dermal mast cells
0.4	0.5	++	++
0.4	1	++	++
0.4	2	++	++
0.4	4	+	+
0.6	0.5	++	++
0.6	1	++	+
0.6	2	++	++
0.6	4	+	+
0.8	0.5	++	+
0.8	1	++	+
0.8	2	++	++
0.8	4	+	+
1.6	0.5	(+)	+
1.6	1	(+)	+
1.6	2	(+)	+
1.6	4	(+)	+

All combinations tested, with the exceptions given below, produced an acceptable tissue fixation in both tissues. The tissues lacked the hardness encountered in preparations fixed in formaldehyde without additives. In addition formaldehyde evidently eliminated the bloating effect of acetic acid. Preparations fixed in the 0.4 or 0.6 per cent formaldehyde and 2 or 4 per cent acetic acid mixtures were nevertheless similar in appearence to preparations fixed in low concentrations of acetic acid only. They were thus bloated, loose and difficult

to cut, and the stained sections lacked a sufficiently detailed histological structure The worst combination in this respect was the mixture of 0 4 per cent formaldehyde and 4 per cent acetic acid

Intestinal mast cells could be demonstrated in preparations fixed in combinations of 0 4 to 0 8 per cent formaldehyde and 0 5 to 2 per cent acetic acid After fixation in all tested mixtures within these ranges, the number of demonstrable cells was approximately the same, the granules were distinctly outlined and displayed an intense metachromasia which often had a reddish tint Specimens fixed in combinations containing 4 per cent acetic acid contained fewer cells with paler and less contrasty metachromasia The same was the case with preparations fixed in mixtures containing 1 6 per cent formaldehyde

Dermal mast cells with heavily granulated cytoplasm and dark violet metachromasia could be demonstrated after fixation in all tested solutions with one exception This was the mixture containing 0 4 per cent formaldehyde and 4 per cent acetic acid which produced mast cells with similar appearance as after fixation in acetic acid alone The cells thus treated showed a reddish metachromasia and the cell margins were indistinct

This experiment revealed that the poor overall tissue fixation resulting from the use of acetic acid alone in low concentrations can be improved by the addition of small amounts of formaldehyde Mast cells having distinctly outlined margins and granules with intense metachromasia can be demonstrated after fixation in mixtures of 0 4 to 0 8 per cent formaldehyde and 0 5 to 2 per cent acetic acid Provided the acetic acid is not allowed to exceed a concentration of 1 per cent, the tissue fixation will be of a quality near that of most commonly used fixatives Of all tested combinations, that containing 0 6 per cent formaldehyde and 0 5 per cent acetic acid was chosen, perhaps somewhat arbitrarily as an alternative fixative for mast cells This solution is approximately blood isotonic (osmolar concentration 300 milliosmoles per litre, i e = 0 95 per cent NaCl) and its pH is 2 9 This Isotonic Formaldehyde-Acetic Acid mixture will hereafter be abbreviated IFAA The number of intestinal mast cells demonstrable after fixation in this solution is of the same order of magnitude as after fixation in any of the lead containing fixatives In addition the tissue fixation is generally better and the tissues lack the coarse precipitations often encountered after the use of lead containing fixatives

4 *Distribution and Morphology of Mast Cells in the Rat after Fixation in IFAA and 4 per cent Formaldehyde*

Comparison of adjacent tissue pieces fixed in IFAA and 4 per cent formaldehyde revealed that the two fixation methods yielded similar results regarding the approximate quantity distribution and morphology of mast cells in all the examined organs except the gastrointestinal

tract Mast cells in H AA fixed tissues seemed however to maintain their shape better, their margins being smoother and fewer being ruptured with scattering of the granules Such mast cells as could be demonstrated well with both fixatives agreed with the classical appearance of mast cells in the rat They were generally large rounded or oval containing granules showing an intense violet metachromasia usually filling the entire cell and obscuring the nucleus Such cells most numerous in tongue ear and skin also occurred in great quantities in the loose subcutaneous tissue and in the mesenterium in agreement with other reports (cf *Michels* 1938 *Riley* 1959) The thymus and lymph nodes contained a moderate number of mast cells most of which were located in the capsular connective tissue The spleen appeared completely devoid of mast cells apart from occasional cells encountered in the capsule

The distal part of the oesophagus contained a moderate number of mast cells evenly distributed in the submucous layer and smaller numbers between the muscular and in the subserous layers Mast cells with similar distribution and morphology could also be demonstrated in the submucous and external wall layers of both the forestomach and the glandular stomach In addition occasional mast cells were encountered in the subserous coat of the remainder of the alimentary tract Mast cells at all these locations were equally well demonstrated after fixation in H AA and 4 per cent formaldehyde and their morphological appearance agreed with that of mast cells in other tissues as described above

Tissues from different parts of the gastrointestinal tract fixed in 4 per cent formaldehyde appeared completely devoid of mast cells apart from the cells occurring in the adjacent mesenterial tissue and the occasional cells encountered in the serous or subserous coats The specimens fixed in H AA on the other hand displayed a considerable number of mast cells located in the mucosa These cells differed from mast cells elsewhere both morphologically and in staining properties They were generally smaller had greatly varying shapes and often exhibited processes They were less densely granulated the rounded or oval nucleus was often clearly visible and not obscured by granules The metachromatic colour of the granules was as a rule reddish rather than violet The mucosal mast cells were most numerous in jejunum and ileum where they occurred in quantities comparable to the number of mast cells in ear or even tongue The duodenal and colonic mucosa also contained a considerable number of mast cells though seemingly fewer than the jejunal or ileal mucosa The mucosa of the glandular part of the stomach contained a smaller number of mast cells than the duodenal mucosa The mucosal mast cells of the stomach were generally concentrated in the superficial part of the mucosa in agreement with the findings of *Rasanen* (1960) The thin submucosal coat of the bowel contained a variable number of mast cells always much

smaller than in the mucosa The mucosal mast cells were generally confined to the lamina propria where they were evenly distributed from the bottom of the glandular crypts to the top of the villi In addition mast cells were sometimes observed within the epithelial tissue where they seemed to be interspersed among the epithelial cells In some instances mast cells were observed even in the most superficial parts of the epithelium lining the glandular lumina No obvious sex differences were noted with respect to distribution morphology or tinctorial properties of mucosal mast cells Nor were there any differences between the two strains studied

DISCUSSION

The commonly accepted difinition of mast cells is based on the metachromasia of their granules towards the basic thiazine dyes Although the validity of such a definition can be questioned (*Simpson* 1963) most workers use only methods based on this property for demonstration of mast cells in tissues Toluidine blue is the most frequently used dye Dye concentration type of solvent and pH of staining solution however are subject to wide variations A strong alcohol resistant metachromasia is indicative of the presence of acidic mucopolysaccharides but the specificity of the staining is partly dependent on the pH of the staining solution (*Spicer* 1962) The technique used in the present investigation is thus probably not specific for strongly sulphated mucopolysaccharides such as heparin but the metachromasia could be due to other mucopolysaccharides as well A detailed study of the staining properties of intestinal mast cells is in progress and will be published separately (*Enerback* 1966)

If a content of metachromatic cytoplasmic granules is accepted as a definition of mast cells two types can be distinguished in the rat One type is characterized by great resistance to fixatives including 4 per cent formaldehyde Most of the cells belonging to this type are large containing densely packed intensely violet metachromatic granules The nucleus is as rule rendered indistinguishable by the dense granulation These cells occur in the connective tissue of most organs except the mucous membranes lining the gastrointestinal tract and are most numerous in tongue and skin To this category belong the cells encountered in the mesentery and subcutaneous tissue

The second type of mast cell can be demonstrated only after fixation in a few out of many tested fixatives Carnoy s solution various solutions of lead salts and in low concentrations of formaldehyde and acetic acid Mast cells of this type occur in the gastrointestinal mucous membranes and in smaller quantities in the submucous coats of the bowel and have properties similar to cells described as atypical mast cells (cf *Maximow* 1906 *Michels* 1938) Thus these cells are smaller vary widely in shape and are more sparsely granulated than other mast

cells. Their granules stain reddish rather than violet with toluidine blue

Considering that these two types of mast cell are *encountered* at different sites, and that organs very rich in the first type seem entirely devoid of the second type, these two types of cell would appear actually to represent dissimilar cell types rather than different developmental stages of the same cell The atypical mast cells are apparently confined to the mucosa of the stomach and bowel and to the submucous coat of the bowel, it is rather interesting that such cells as occur in the submucous layer of the gastric wall seem to be of the formaldehyde resistant mast cell type encountered in other organs However, on the basis of available data, the possibility cannot be ruled out that other organs too may contain a small proportion of these atypical mast cells Mast cells exhibit some variations in size and shape as well as in granulation density within organs—skin mast cells for example—and hence morphological criteria are unlikely to suffice for a differentiation of these cell types

A method of differentiating between these two types of cell could be based on their reactions towards fixatives In experiments such as those reported here the observed effect is the sum of fixation and subsequent histological procedure, which cautions against making comparisons between different sets of data Obviously however, in tissues otherwise treated identically, intestinal mast cells cannot be demonstrated after many fixations which allow the demonstration of *dermal mast cells*

In most instances fixation of acid mucopolysaccharides involves their precipitation in the tissues (*Szirmay* 1963) In the case of mast cells fixation could be achieved by precipitation of either the acid mucopolysaccharides or associated proteins Failure to demonstrate mast cells could then be due either to dissolution of non-precipitated stainable material or to interference with staining by some component of the fixative through blocking of dye binding groups

The preservative effect on intest[illegible]
and solutions [illegible]
effects on a [illegible] ...avy metals can precipitate both [illegible] ...polysaccharides and proteins (*Curran* 1961) Similarly, Carnoy's solution is generally considered an adequate fixative for acid mucopolysaccharides and its effect has been related to its acidity, facilitating ionic linkage to proteins (*Szirmay* 1963)

Ethanol, at least absolute, is considered to precipitate most acid mucopolysaccharides in tissues (*Curran* 1961) Its effect is reversible however, which may be the reason why the present technique fails to demonstrate intestinal mast cell granules after ethanol fixation The findings of *Dalgaard & Dalgaard* (1948) indicate that [illegible] ...st cell granules can be stained in [illegible] ...d
material [illegible]

The finding that formaldehyde in concentrations lower than those ordinarily used for fixation can preserve the stainability of intestinal mast cells while higher concentrations fail to do so is of some interest. Formaldehyde is an adequate fixative for many acid mucopolysaccharides and its effects are probably dependent on fixation of associated proteins (*Curran* 1964). Some mucopolysaccharides are however extracted by formaldehyde (*Szirmay* 1963). As far as is known formaldehyde is unable to react with anionic tissue polysaccharides (*Schubert & Hamerman* 1956). On the contrary formaldehyde in some conditions enhances the basophilia of mast cell granules (*Spicer* 1963). Hence the failure of higher concentrations of formaldehyde to preserve the stainability of intestinal mast cell granules most probably involves a loss of stainable material through dissolution. Formaldehyde exerts its effect on proteins partly by combining with basic groups with a resulting decrease in affinity to acid dyes. Similarly blocking of basic protein groups would interfere with ionic linkages between acid mucopolysacharides and proteins and could thus be the reason for a dissolution of stainable material in intestinal mast cell granules after fixation in higher concentrations of formaldehyde.

Thus whichever mechanism is involved the differences in reactions towards fixatives between intestinal and other mast cells in the rat suggests that the chemical composition of their granules differs.

SUMMARY

The gastrointestinal mucosa of the rat contains a great number of mast cells which in many respects differ from mast cells elsewhere. The mucosal mast cells are smaller, not so densely granulated and stain reddish rather than violet with toluidine blue at pH 4. The mucosal mast cells moreover lack the characteristic resistance to fixatives typical of other mast cells in the rat. In particular intestinal mast cells cannot be demonstrated after fixation in 4 per cent formaldehyde but their stainability with toluidine blue is well preserved after fixation in 0.4–0.8 per cent formaldehyde solutions. This fact together with the behaviour of intestinal mast cells after treatment with other fixatives indicates that the granules of intestinal mast cells are more easily dissolved during the histological process than those of other mast cells. Based on these observations a fixative for mast cells has been formulated containing 0.6 per cent formaldehyde and 0.5 per cent acetic acid. This fixative can be used as an alternative to Carnoy's solution or fixatives containing lead salts. It gives a good preservation of stainability with toluidine blue of both types of mast cell in the rat.

REFERENCES

Ballantyne F. N.: Differentiation of Plasma Cells from Mast Cells in the Intestinal Mucosa of the White Rat. Canad. M. A. J. *21*: 135, 193, 1929.

Curran, R R The Histochemistry of Mucopolysaccharides Int Rev Cytol *17* 149 212 1964

Dalgaard F & Dalgaard J B A Histologic and Histochemical Study of Mast Cells and their Contents of Phosphatase Ugesk. f laeger *110* 513 518 1948

Enerbäck L Mast Cells in Rat Gastrointestinal Mucosa Dye binding and metachromatic properties Acta path et microbiol scandinav *66* 303 312 1966

Harbers F Autoradiographie als histochemisches Untersuchungsverfahren In Handbuch der Histochemie, Band 1, Erster Teil Grauman, W & Neuman K, Ed. Gustav Fischer Verlag Stuttgart 1958

Holmgren H & Wilander O Beitrag zur Kenntnis der Chemie und Funktion der Ehrlichschen Mastzellen Ztschr f mikr anat Forsch *42* 242 278 1937

Kelsall M A & Crabb E D Lymphocytes and Mast Cells Williams and Wilkins Company, Baltimore 1959

Levine N D *The Dehydration of Methylene Blue Stained Material Without Loss of Dye* Stain Tech *14* 29-30 1939

Lillie R D Histopathological Technic and Practical Histochemistry The Blakiston Co Inc New York, 1954

Lindholm, S The Occurrence of Connective Tissue Mast Cells in the Alimentary Canal Wall of Hamster and Rat Acta path et microbiol scandinav *45* 328 331 1960

Maximow A Über die Zellformen des lockeren Bindegewebes Arch f mikr Anat (Bonn) *67* 680-757, 1906

Michels N A The Mast Cells in Lower Vertebrates La Cellule *33* 339 462 1923

Michels N A The Mast Cells In Downey's Handbook of Haematology *1* 232 372 1938 Republished in Mast Cells and Basophils Padawer J Ed Ann N Y Acad Sci *103* 1 492 1963

Mota I Ferri A G & Yoneda S The Distribution of Mast Cells in the Digestive Tract of Laboratory Animals its bearings on the Problem of the Location of Histamine in the Tissues Quart J micr Sci *97* 251 256 1956

Padawer J Studies on Mammalian Mast Cells Tr N York Acad Sc *56 57* 690 713 1957

Parish W F The Distribution of Mast Cells and the possible Function of some of their Pharmacological Agents In Biological Aspects of Occlusive Vascular Disease Chalmers D G & Gresham, G A Ed The Cambridge University Press, 1964

Riley J F The Mast Cells E & S Livingstone Ltd, Edinburgh and London, *1959*

Romeis B Mikroskopische Technik R Oldenburg München 1948

Räsänen T Mucosal Mast Cells of Rat Stomach Influence of ACTH Cortison and Growth Hormone Gastroenterology *38* 70 75 1960

Räsänen T Quantitative Effects of Some Steroid Hormones on the Mucosal Mast Cells and Tissue Eosinophils of the Rat Stomach Wall Acta Endocr (Kbh) *37* 153 160 1961

Räsänen T Effects of Dexamethasone Prednisolone and Cortisol on Mast Cells and Tissue Eosinophils in Rat Gastric Mucosa Acta Endocr (Kbh) *41* 432 436, 1962

Schubert M & Hamerman D Metachromasia Chemical Theory and Histochemical Use J Histochem Cytochem *4* 159 189 1956

Simpson W L Distribution of Mast Cells as a Function of *Age and Exposure to* Carcinogenic Agents In Mast Cells and Basophils Padawer J Ed Ann N Y Acad Sci *103* 1 492 1963

Spicer S S Histochemical Differentiation of Sulphated Rodent Mucin Ann Histochim *7* 23 28 1962

Spicer S S Histochemical Properties of Mucopolysaccharide and Basic Protein in Mast Cells In Mast Cells and Basophils Padawer *J Ed Ann* N Y Acad Sci *103* 1 492, 1963

Szirmai J A Quantitative Approaches *in the Histochemistry of Mucopolysaccharides* J Histochem Cytochem *11* 24 34 1963

Westphal E Über Mastzellen In Ehrlich P *Farbenanalytische* Untersuchungen zur Histologie und Klinik des Blutes Berlin Hirschwald 1891 Cited by Riley *J F 1959*

Acta path et microbiol scandinav 66 303 312 1966

Institute of Pathology 1 (Head Professor Jan Mellgren),
University of Gothenburg Sweden

MAST CELLS IN RAT GASTROINTESTINAL MUCOSA

2 Dye-Binding and Metachromatic Properties

By

LENNART ENERBÄCK

Received 2 ix 65

Mucosal mast cells in the gastrointestinal tract of the rat differ from mast cells elsewhere One of the most easily recognized differences is that mucosal mast cells lack the resistance towards fixatives typical of other mast cells in the rat In a recent paper (*Enerback* 1966) the effect of fixation on rat mast cells was studied using staining with 0 5 per cent toluidine blue at pH 4 as a means of demonstrating the mast cells The following communication describes the stainability, using different basic dyes and different pH levels, of the two types of mast cell in the rat

MATERIALS AND METHODS

Nine male albino rats of the Sprague Dawley strain (Anticimex AB Stockholm) weighing 147 163 g were used The treatment of the animals and the general histological procedure were those reported earlier (*Enerback* 1966) Tissues from the midportion of the small intestine and from the back skin were fixed for 24 hours in an Isotonic Formaldehyde Acetic acid mixture (IFAA *Enerback* 1966) 4 per cent neutral buffered formaldehyde (FA) Carnoy s fixative (Ca) and in a 4 per cent aqueous solution of lead acetate containing 0 5 per cent acetic acid (Pb) All the material from one rat was embedded in the same paraffin block and was subjected to the same histological procedure Staining was performed on 5 µ sections

Staining Procedures

Thiazine dyes Toluidine blue (Merck AG Darmstadt CI No 52040 2nd Ed) was used in a 0 5 per cent aqueous solution at pH 4 (Mc Ilvaine s citric acid disodium phosphate buffer staining time 45 seconds) at pH 1 5 (potassium chloride hydrochloric acid buffer according to *Clark & Lubb* staining time 30 minutes) and at about pH 0 5 (dye diluted in 0 5 N HCl staining time 30 minutes)

Other sections were stained with an 0 1 per cent aqueous solution at about pH 0 3 (dye diluted in 0 7 N HCl) for 10 minutes followed by rinsing in 0 7 N HCl for 10 minutes This technique was identical to that used for Astra blue and Alcian blue staining (see below) as devised by *Bloom & Kelly* (1960) for Astra blue To check the effect of staining time and dye concentration on dye binding sections were stained in a 0 5 per cent dye solution at pH 0 5 with staining times increasing from 30 seconds to 32 minutes and in dye solutions at pH 0 5 for 30 minutes with dye concentrations increasing from 0 005 per cent to 0 5 per cent

Azure A (G T Gurr Ltd London CI No 52005 2nd Ed) was used in a 0 02 per cent concentration at pH 4 1 5 and 0 5 with the buffers described above

Copper phthalocyanine dyes Staining with Astra blue was performed according to *Bloom & Kelly* (1960) The dye was Astrablau 6 GLL Extra (Bayer AG Leverkusen obtained by courtesy of Bayerkemi AB Göteborg) Two other samples were

Curran R R The Histochemistry of Mucopolysaccharides Int Rev Cytol *17* 149 212 1964

Dalgaard E & Dalgaard J B A Histologic and Histochemical Study of Mast Cells and their Contents of Phosphatase Ugesk f laeger *110* 513 518 1948

Enerback L Mast Cells in Rat Gastrointestinal Mucosa Dye binding and metachromatic properties Acta path et microbiol scandinav *66* 303-312 1966

Harbers E Autoradiographie als histochemisches Untersuchungsverfahren In Handbuch der Histochemie Band 1 Erster Teil Grauman W & Neuman K, Ed Gustav Fischer Verlag Stuttgart 1958

Holmgren HJ & Wilander O Beitrag zur Kenntnis der Chemie und Funktion der Ehrlichschen Mastzellen Ztschr f mikr anat Forsch 42 242 278 1937

Kelsall M A & Crabb E D Lymphocytes and Mast Cells Williams and Wilkins Company Baltimore 1959

Levine N D The Dehydration of Methylene Blue Stained Material Without Loss of Dye Stain Tech *14* 29 30 1939

Lillie R D Histopathological Technic and Practical Histochemistry The Blakiston Co Inc New York 1954

Lindholm S The Occurrence of Connective Tissue Mast Cells in the Alimentary Canal Wall of Hamster and Rat Acta path et microbiol scand nav 48 328-331 1960

Maximow A Uber die Zellformen des lockeren Bindegewebes Arch f mikr Anat (Bonn) 67 680-757 1906

Michels N A The Mast Cells in Lower Vertebrates La Cellule *33* 339 462 1923

Michels N A The Mast Cells In Downey s Handbook of Haematology *1* 232-372 [illegible]

[illegible]

1957

Parish W E The Distribution of Mast Cells and the possible Function of some of their Pharmacological Agents In Biological Aspects of Occlusive Vascular Disease Chalmers D G & Gresham G A Ed The Cambridge University Press, 1964

Riley J F The Mast Cells E & S Livingstone Ltd Edinburgh and London 1959

Romeis B Mikroskopische Technik R Oldenbourg Munchen 1948

Rasanen T Mucosal Mast Cells of Rat Stomach Influence of ACTH Cortison and Growth Hormone Gastroenterology 38 70-75 1960

Rasanen T Quantitative Effects of Some Steroid Hormones on the Mucosal Mast Cells and Tissue Eosinophils of the Rat Stomach Wall Acta Endocr (Kbh) 37 153 160 1961

Rasanen T Effects of Dexamethasone Prednisolone and Cortisol on Mast Cells and Tissue Eosinophils in Rat Gastric Mucosa Acta Endocr (Kbh) *41* 432 436 1962

[illegible] *Hamerman D* Metachromasia Chemical Theory and Histochemical [illegible]

[illegible] n of Age and Exposure to [illegible] Padawer J Ed Ann N Y [illegible]

[illegible] to lent Mucins Ann Hist [illegible]

chim [illegible]

Spicer S S Histochemical Properties of Mucopolysaccharide and Basic Protein in Mast Cells In Mast Cells and Basophils Padawer J Ed Ann N Y Acad Sci *103* 1 492 1963

Szirmai J A Quantitative Approaches in the Histochemistry of Mucopolysaccharides J Histochem Cytochem *11* 24 34 1963

Westphal E Uber Mastzellen In Ehrlich P Farbenanalytische Untersuchungen zur Histologie und Klinik des Blutes Berlin Hirschwald 1891 Cited by Riley J F, 1959

RESULTS

The results of the different staining procedures are recorded in Tables 1 to 4 where the staining intensities have been arbitrarily quantitated according to the following schedule

++ = many strongly stained mast cells
+ = many weakly stained mast cells
(+) = few weakly stained mast cells, some sections lacking stained mast cells altogether
0 = no stained mast cells in any examined section

Thiazine Dyes

Both types of mast cell were found to stain with toluidine blue and azure A at pH 0.5. Toluidine blue generally stained the dermal mast cells a strong dark violet colour and the intestinal mast cells a more

TABLE 1
Staining of Intestinal and Dermal Mast Cells with Thiazine Dyes at Different pH Levels

Fixation	Tissue	Toluidine blue 0.5 per cent			Azure A 0.02 per cent		
		pH			pH		
		0.5	1.5	4	0.5	1.5	4
IFAA	Intestine	++	+	++	++	+	0*
	Skin	++	++	++	++	++	++
FA	Intestine	0	0	0	0	0	0
	Skin	++	++	++	++	++	++
Ca	Intestine	++	+	+	++	(+)	0*
	Skin	++	++	++	++	++	++
Pb	Intestine	++	+	++	++	(+)	0*
	Skin	++	++	++	++	++	++

* Increase of dye concentration to 0.08 per cent resulted in ++

TABLE 2
Staining of Intestinal and Dermal Mast Cells with Toluidine Blue at pH 0.5. Staining Time 30 Minutes. Effect of Dye Concentration

Fixation	Tissue	Per cent dye concentration				
		0.5	0.225	0.05	0.0225	0.005
IFAA	Intestine	++	++	0	0	0
	Skin	++	++	++	++	++
FA	Intestine	0	0	0	0	0
	Skin	++	++	++	++	++
Ca	Intestine	++	++	+	0	0
	Skin	++	++	++	++	++
Pb	Intestine	++	++	+	+	0
	Skin	++	++	++	++	++

TABLE 3

Staining of Intestinal and Dermal Mast Cells with 0.5 per cent Toluidine Blue at pH 0.5. Effect of Staining Time

Fixation	Tissue	Staining time			
		32 min	8 min	2 min	30 sec
IFAA	Intestine	++	+	+	0
	Skin	++	++	++	++
FA	Intestine	0	0	0	0
	Skin	++	++	++	++
Ca	Intestine	++	++	+	+
	Skin	++	++	++	++
Pb	Intestine	++	++	++	+
	Skin	++	++	++	++

TABLE 4

Staining of Intestinal and Dermal Mast Cells at pH 0.3 (0.7 N HCl) Followed by Immersion of Sections in 0.7 N HCl. Dye Concentration 0.1 per cent

Fixation	Tissue	Toluidine blue	Astra blue	Astra blue safranin
IFAA	Intestine	0	+	B
	Skin	+	(+)	BR
FA	Intestine	0	0	0
	Skin	+	+	BR
Ca	Intestine	0	++	B
	Skin	++	++	BR
Pb	Intestine	0	++	B
	Skin	++	+	BR

* B = blue R = red

reddish metachromatic colour. The staining with azure A at pH 0.5 and 1.5 was almost pure blue both in intestinal and dermal mast cells without obvious metachromatic shift. Intestinal mast cells lacked stainability with azure A at pH 4 but the dermal mast cells stained strongly at this pH with a dark violet obviously metachromatic colour. The absence of staining of intestinal mast cells with azure A at pH 4 was unexpected in view of the staining at pH 0.5. The intestinal mast cells could however be stained at pH 4 if the dye concentration was increased to 0.08 per cent. Moreover intestinal mast cells were stained strongly metachromatic with azure A at pH 4. Intestinal mast cells did not stain with 0.1 per cent toluidine blue at pH 0.3 but the dermal mast cells stained strongly (see Table 4).

These results indicate a weaker binding of thiazine dyes in intestinal than in dermal mast cells. This was further illustrated in the trial with different staining times and different dye concentrations. It was found as shown in Tables 2 and 3 that the staining of intestinal mast cells

could be extinguished by decreasing the dye concentration or staining time at pH 0 5 without marked decrease in staining intensity of dermal mast cells Furthermore intestinal mast cells could be stained with azure A at pH 4 when the dye concentration was increased to 0 08 per cent

The most obvious effect of fixation was that intestine fixed in 4 per cent formaldehyde did not show any mast cells at all with any staining method The other fixatives gave good preservation of stainability of intestinal mast cells with toluidine blue and azure A Toluidine blue at pH 4 gave however somewhat weaker staining of mast cells fixed in Carnoy s solution than of those fixed in IFAA or lead acetate In addition the trial with different dye concentrations and staining times revealed that a higher dye concentration or a longer staining time was needed for the demonstration of mast cell staining with toluidine blue in IFAA fixed sections than in those fixed in Carnoy or lead acetate

Copper Phthalocyanine Dyes

In Table 4 the results of Astra blue staining at pH 0 3 alone or combined with safranin have been recorded Staining with Alcian blue at the same pH gave similar results Again it was found that no intestinal mast cells were stained after fixation in 4 per cent formaldehyde Intestine fixed in the other solutions contained approximately the same number of mast cells as that seen after staining with thiazine dyes The intestinal mast cell granules stained pure blue while other structures including mast cell nuclei remained unstained Intestinal mast cells fixed in Carnoy or lead acetate were somewhat stronger stained than the mast cells of IFAA fixed material

The dermal mast cells reacted differently to Astra blue however In IFAA fixed sections only a few often weakly stained mast cells could be observed Formaldehyde fixed sections also showed very weakly stained mast cells but the quantity of cells was roughly the same as in sections stained with thiazine dyes On the other hand the Carnoy fixed sections revealed strongly stained mast cells in approximately the same quantity as in sections stained with thiazine dyes Lead fixed skin preparations contained approximately the same number of mast cells as the Carnoy fixed sections but were more weakly stained The difference in affinity to copper phthalocyanine dyes between intestinal and dermal mast cells was further demonstrated in the Astra blue safranin stained sections Here intestinal mast cells stained pure blue On the other hand the dermal preparations contained a mixture of blue and red staining cells and even mast cells in which some of the granules where coloured blue and some red Effects of fixation were similar to those observed after staining with Astra blue alone Thus in IFAA fixed sections the majority of the mast cells stained red and in Carnoy fixed skin most of the mast cells stained blue

Both intestinal and dermal mast cells were found to be periodic acid Schiff unreactive after all fixations

DISCUSSION

The metachromasia of mast cell granules has been attributed to their content of sulphated mucopolysaccharides *Jorpes Holmgren & Wilander* (1937) demonstrated a close correlation between the quantity of mast cells and the heparin content in tissues and since then it has been widely accepted that mast cells contain heparin Not until recently however has it become possible to establish the types of mucopolysaccharides present in mast cells In the case of peritoneal mast cells in the rat it has thus been demonstrated that the mucopolysaccharide is solely heparin (*Schiller & Dorfman* 1959 *Bloom & Ringertz* 1960) Mast cell tumours however contain other mucopolysaccharides as well (*Ringertz* 1963)

A metachromatic reaction with cationic dyes is caused by a number of high molecular polyanions (*Schubert & Hamerman* 1957) the most important such substances occurring in tissues being sulphated mucopolysaccharides nucleic acids and certain high molecular weight lipids The principle of staining at controlled pH levels in order to distinguish between different mucopolysaccharides on the basis of their relative acidity was introduced by *Dempsey et al* (1947), who noted that some structures known to contain sulphate groups maintained their basophilia at low pH This principle has been utilized by *Spicer* (1960) to differentiate acid mucopolysaccharides Similarly *Bloom & Kelly* (1960) demonstrated that mast cells could be stained with the basic copper phthalocyanine dye Astra blue at pH 0 2 when all other tissue constituents failed to stain except some epithelial mucins and cartilage They assumed that the staining was due to sulphate groups being the only acid radicals likely to be dissociated at this low pH The blocking of Astra blue staining with cetylpyridinium chloride provided additional evidence that the dye combines with sulphate groups

The specificity for sulphate groups of copper phthalocyanine staining at low pH was confirmed by *Scott Quintarelli & Dellovo* (1964) and by *Quintarelli Scott & Dellovo* (1964 a and b) who extensively studied the chemical and histochemical properties of Alcian blue Their results indicate that Alcian blue combines with tissue sulphate groups through ionic linkages Artificial sulphation of epithelial mucins which did not stain with azure A or Alcian blue at pH below 1 in their native state induced such staining Moreover desulphation of sulphated structures reduced or abolished Alcian blue staining at pH 0 4

In the light of the foregoing binding of copper phthalocyanine and thiazine dyes at low pH in intestinal and dermal mast cells indicates the presence of sulphated mucopolysaccharides in both types of cell On the other hand intestinal and dermal mast cells showed distinct

differences in affinity to the two types of dye Thus intestinal mast cells showed a weaker affinity to thiazine dyes than dermal mast cells This was visualized by the lack of staining at pH 0 3 when dermal mast cells stained strongly and by the slower dye binding rate and the need of higher dye concentrations for the demonstration of dye binding and metachromasia in intestinal mast cells

On the other hand intestinal mast cells seemed to have a stronger affinity for Astra blue than dermal mast cells This was most obvious in tissues fixed in IFAA where only a small amount of dermal mast cells was stained but apparently all the intestinal mast cells Similarly when safranin was applied after Astra blue approximately the same number of mast cells were stained as after toluidine blue but most of the cells stained red from safranin In intestinal mast cells however no red staining cells could be observed after staining with the Astra blue safranin sequence

The significance of these differences between intestinal and dermal mast cells cannot be fully evaluated on the basis of available data It seems logical however to assume that they reflect differences in physical properties or chemical composition of the granules of the two types of cell The results adequately explain why the presence of mast cells in rat intestinal mucosa has sometimes been overlooked even when the fixation technique used was sufficient for the preservation of stainable material in these cells

The reported results suggest that even if copper phthalocyanine and thiazine dyes at a sufficiently low pH selectively demonstrate the presence of sulphate groups in tissues the two types of dye may nevertheless differ in their affinity for sulphated structures One experiment with artificial sulphation of epithelial mucins reported by *Quintarelli Scott & Devello* (1964) points in the same direction In general sulphation of such mucins gave rise to a strong Alcian blue staining at pH 0 4 but one of the tested mucins (mouse sublingual mucin) reacted differently Here sulphation according to the standard technique gave rise to a strong azure A staining at pH 1 but the mucin did not stain with Alcian blue at pH 0 4 even if the sulphation time was prolonged If however the concentration of sulphuric acid was lowered the mucin stained strongly with Alcian blue at pH 0 4 with preservation of azurophilia

Differences between Alcian blue and azure A in affinity to sulphated structures have also been reported by *Spicer* (1959 1960 1962) who used Alcian blue at pH 2 4 only Among structures with a known or presumed content of sulphate groups those which could be stained with azure A at a lower pH had a comparatively weaker affinity to Alcian blue than those which stained with azure A at a higher pH only Sulphation of epithelial mucins resulted in a strong azurophilia but a reduction of Alcian blue staining at pH 2 4 Desulphation of strongly sulphated structures reduced the azurophilia and increased Alcian

blue affinity On the basis of these results *Spicer* suggested (1962) that the density or spacing of the sulphate groups on the mucopolysaccharide units might be responsible for these differences in affinity of the two types of dye

In an attempt further to clarify this matter experiments with radio-autography after injection of $Na_2{}^{35}SO_4$ are in progress (*Enerbäck & Knutson*, to be published) The hitherto available data indicate an uptake of ^{35}S both in the intestinal and the dermal mast cells Due to the high activity of ^{35}S in intestinal epithelium and stroma shortly after injection, the uptake in intestinal mast cells could not be evaluated until 24 hours after injection At this time, however, the activity was much lower in intestinal than in dermal mast cells This might imply either a lower content of sulphate in intestinal mast cells and/or different turnover rates of sulphate in the two types of cell

The results of the present investigation further demonstrate that the metachromatic shade of mast cell granules is a quality which must be interpreted with caution Thus 0 5 per cent toluidine blue generally stained both intestinal and dermal mast cells in an obviously metachromatic shade but the intestinal mast cells often appeared redder 0 02 per cent azure A at pH 0 5 and 1 5 on the other hand stained both intestinal and dermal mast cells almost pure blue without obvious metachromasia *Kramer & Windrum* demonstrated (1955) that overstaining of strongly metachromatic structures may make them appear almost orthochromatic, a fact easily confirmed in the case of mast cells In addition, *Radden* (1960) reported that in tissues stained in dilute toluidine blue solution and examined in the wet state the mast cells at first appeared orthochromatic and turned metachromatic with increasing staining times The tinctorial properties of the metachromasia are obviously partly influenced by dye concentration and staining time, and since some bound dye is removed during dehydration, at least in the case of toluidine blue variations in the dehydration procedure can probably also influence the metachromatic shade

Both dermal and intestinal mast cells were found to be PAS negative This finding is in agreement with the report of *Spicer* (1963) who found mast cells to lack, or have weak affinity for fuchsin sulphurous acid after periodic acid oxidation Similarly, it appears well established that highly sulphated mucopolysaccharides do not contribute to PAS staining (cf Curran 1964) On the other hand, differences in PAS-reactivity have been reported to occur among mast cells within the same individual or tissue, in mast cells of different species and in granules of a single mast cell (cf *Kelsall & Crabb* 1959) *Jorpes, Werner & Aberg* (1948) found PAS-positive mast cells in young rats and attributed the PAS-reaction to heparin monosulphuric acid or some other heparin precursor

The most conspicuous effect of fixation noted in the present investigation was the complete absence of mast cell staining whichever

method was used, in the intestinal preparations fixed in 4 per cent formaldehyde

The previously reported differences, with respect to morphological appearance and reactions upon fixation, between intestinal and other mast cells in the rat (*Enerbäck* 1966) have thus been found to have parallels in staining properties The commonly accepted definition of mast cells as connective tissue cells containing metachromatic cytoplasmic granules is wide enough to include cells with different chemical composition and function Differences, such as those reported previously and in the present communication, between mast cells at different sites in the same species might therefore have important implications even if the factors responsible for the differences at present cannot be fully elucidated Hence experiments are in progress by which further to characterize these two types of cell

SUMMARY

Mast cells in rat intestinal mucosa differ in staining properties from those of skin Intestinal mast cells although staining with thiazine dyes at pH 0 5 have lower affinity for these dyes than dermal mast cells This was demonstrated by lack of staining with toluidine blue of intestinal mast cells at pH 0 3 when dermal mast cells stained strongly Similarly the staining of intestinal mast cells at pH 0 5 could be extinguished by decreasing the dye concentration or staining time without a substantial loss of staining intensity of dermal mast cells

On the other hand basic copper phthalocyanine dyes such as Astra blue seem to have stronger affinity for intestinal than for dermal mast cells Thus intestinal mast cells stained strongly with Astra blue at pH 0 3 in an Astra blue safranin sequence On the other hand, dermal mast cells stained partly blue from Astra blue and partly red from safranin the proportion of cells staining blue being dependent on the type of fixative used

These differences in staining properties are discussed and related to previously reported differences between the two types of cell with respect to morphological appearance and reactions upon fixation

REFERENCES

Bloom G & Kelly J W [illegible]
ing Properties espe [illegible]

Bloom G & Ringert [illegible]
and Mouse Arkiv 1 [illegible]

Curran R C The Histochemistry of Mucopolysaccharides Int Rev Cytol *17* 149 212 1964

Dempsey F W Bunting H Singer M & Wislocki C B The Dyebinding Capacity and other Chemo histological Properties of Mammalian Mucopolysaccharides Anat Rec *98* 417–429 1947

Enerbäck L Mast Cells in Rat Gastrointestinal Mucosa 1 Effects of Fixation Acta path et microbiol scandinav *66* 289 302 1966

Enerback L & Knutson F To be published
Jorpes F Holmgren Hj & Wilander O Uber das Vorkommen von Heparin in den Gefasswanden und in den Augen Ein Beitrag zur Physiologie der Ehrlichschen Mastzellen Ztschr f mikr anat Forsch 42 279–301 1937
Jorpes J F Werner G & Aberg B The Fuchsin sulphurous Acid Test after Periodate Oxidation of Heparin and Allied Polysaccharides J Biol Chem 176 277 282 1948
Kramer H & Windrum G M The Metachromatic Staining Reaction. J Histochem Cytochem 3 227 237 1955
Quintarelli G Scott J F & Dellello M C The Chemical and Histochemical Properties of Alcian Blue II Dye binding of Tissue Polyanions Histochemie 4 86 98 1964
Quintarelli G., Scott J E & Dellello M C The Chemical and Histochemical Properties of Alcian Blue III Chemical Blocking and Unblocking Histochemie 4 99 112 1964
Ringertz N R Polysaccharides of Neoplastic Mast Cells In Mast Cells and Basophils Padawer, J Ed Ann N Y Acad Sci 103 1 492 1963
Schiller S & Dorfman A The Isolation of Heparin from Mast Cells of the Normal Rat Biochim Biophys Acta 31 278 280 1959
Schubert M & Hamerman D Metachromasia Chemical Theory and Histochemical Use J Histochem 4 159 189 1956
Scott J E Quintarelli G Dellello M C The Chemical and Histochemical Properties of Alcian Blue I The Mechanism of Alcian Blue Staining Histochemie 4 73–85 1964
Spicer S S Swarm R L & Burtner H J Comparison of Basophilia with S35 Label in Normal and Methylated Mucopolysaccharides Lab Invest 10 256 264 1959
Spicer S S A Correlative Study of the Histochemical Properties of Rodent Acid Mucopolysaccharides J Histochem Cytochem 8 18 34 1960
Spicer S S Histochemical Differentiation of Rodent Acid Mucins Ann Histochim 7 23 28 1962
Spicer S S Histochemical Properties of Mucopolysaccharide and Basic Protein in Mast Cells In Mast Cells and Basophils Padawer J Ed Ann N Y Acad Sci 103 1 492 1963

Acta path et microbiol scandinav 66 313 322 1966

Institute of Pathology I (Head Professor Jan Mellgren M D),
University of Gothenburg Sweden

MAST CELLS IN RAT GASTROINTESTINAL MUCOSA

3 *Reactivity towards Compound 48/80*

By

LENNART ENERBÄCK

Received 21 xii 65

The gastrointestinal mucosa of the rat contains a large number of granulated cells satisfying the usually accepted definition of mast cells based on their granular dye binding properties These cells do however, differ from mast cells in other tissues of this species both morphologically and histochemically (*Enerbäck* 1966 1966a) Similar differences are known to exist in mast cells between species but their biological significance if any, is unknown Efforts have therefore been made further to characterize these two cell types in the rat The following communication will describe differences in reactivity towards Compound 48 80 between the two types of cell

MATERIALS AND METHODS

Thirty male albino rats of the Sprague Dawley strain (Anticimex AB Stockholm) weighing 126 213 g at the start of the experiments were used They were divided into 5 groups each with six rats of matching body weights Two of the groups served as controls the others received 48/80 [1] Two types of experiments were performed

Subacute Experiment

Six rats received the histamine liberator Compound 48 80 dissolved in saline intra peritoneally twice daily for 5 days starting with 0 1 mg/100 g with daily increments of 0 1 mg/100 g On the fifth day they were given one injection of 0 5 mg/100 g and killed by decapitation 4 hours later Six controls received the same volumes of saline intraperitoneally at the same times The scheme was identical to that used by *Riley & West* (1955) (In another experiment on 5 rats a further increase in 48 80 dosage to 1 0 mg/100 g on the sixth day was fatal within 30 to 60 minutes) The rats receiving up to 0 5 mg 48/80 were autopsied the following tissues being saved for histological study dorsal skin ears lung thymus spleen liver oesophagus stomach duodenum jejunum ileum and colon The pieces from the digestive tract were cut as decribed previously (*Enerbäck* 1966)

Acute Experiment

Six rats were given 0 2 and six 0 8 mg/100 g of 48 80 in saline in a tail vein Rats receiving the lower dose were killed after 15 minutes The others died within 20 seconds to 2½ minutes and were dissected immediately The six controls were

I am deeply indebted to Mrs *Kerstin Bresky* and Mrs *Gun Augustsson* for skilful technical assistance

[1] The 48 80 preparation used was a generous gift from Research Director B Hogberg M D AB Leo Hälsingborg Sweden

injected with saline intravenously in the same volume and killed after 15 minutes Spread preparations were prepared from the mesentery and pieces of dorsal skin, tongue and duodenum were saved for histological study

Histotechnical procedures Spread preparations air dried for 30 minutes at 60° C, were passed through alcohols to xylene and rehydrated by passage through alcohols to water They were then stained with 0 5 per cent toluidine blue at pH 4 for 45 seconds, dehydrated and mounted in Diatex® without cover glasses Tissue pieces were fixed in an isotonic formaldehyde-acetic acid mixture (*IIAA; Enerbäck* 1966) embedded in paraffin and stained with toluidine blue at pH 4 and pH 0 5 Details of these methods have been given previously (*Enerbäck* 1966 a)

Mast cell quantitation Mast cells were counted in 5 *μ* sections of intestine, skin and tongue, stained at pH 0 5 A 40 × objective and an 8 × ocular were used giving visual fields of 0 113 mm² Counts were made in the intestinal mucosa where the specific type of mucosal mast cell is approximately uniformly distributed The mucosal fields, selected at random, bordered against the thin submucous coat 10 fields in each of two sections from each specimen were counted

Skin mast cells were counted in a similar manner Counts were made in the basal part of the corium where mast cells are concentrated The fields were distributed in a random manner their borders facing the muscular sheath which in dorsal rat skin is situated immediately below the corium In ears, where mast cells are approximately uniformly distributed counts were made in fields across the entire section In ears and skin, 10 20 fields were counted in each of two sections from each specimen

The cells were counted in a standardized manner and counting of fragments of cells was avoided In the case of intestine this was easily accomplished by counting nucleated cells only The mast cell nuclei were as a rule not covered by granules and either faintly stained or could be seen as unstained spots within the cells The mast cell quantity was expressed as the mean number of cells per field from each specimen For statistical treatment of the data Wilcoxon s ranking test was used (*Wilcoxon* 1947)

The apparent mast cell quantities thus calculated are subject to both unsystematic and systematic errors The influence of factors belonging to the former category, such as variations in true section thickness state of section hydration etc, are obviously randomized by experimental design and statistical treatment The major systematic errors are variations in cell volume and in the relation between cell volume and tissue volume This means that an apparent increase in cell number per unit volume of tissue can be due to an increase in cell volume or the cells could be pushed together by a decrease in tissue volume due to shrinkage The influence of cell [illegible]
remai [illegible]
To c [illegible]
ment [illegible]
acute experiment mast cells were counted in the duodenal mucosa using a 100 × immersion objective and an 8× ocular The sections were stained at pH 4 Cells with distinctly stained nuclei were counted in 10 random fields from the basal part of the mucosa in each specimen The smallest and largest nuclear diameters were measured in 20 random nuclei in each section In addition the areas of the mucosal cross section were measured planimetrically

RESULTS

Subacute Experiment

After the injection of 48 80 the rats behaved as described by *Riley* (1959) The first two doses of 0 1 mg had no apparent effects Few minutes after the first injection of 0 2 mg, however, the rats exhibited signs of shock and gradually developed strong cyanosis on the ears and paws They gradually recovered after about 15 minutes and appeared normal after one hour Subsequent injections up to the second dose of 0 4 mg gave less obvious effects The injection of 0 5 mg resulted in a slight reaction after 3 4 minutes The rats lay down,



Figs 1 2

Fig 1 Sections from skin Toluidine blue pH 0 5 130 × a) Control Note the large number of densely granulated and heavily stained mast cells b) Rat given repeated injections of 48/80 for 5 days Mast cells are almost completely absent only a few weakly stained cells with few granules remain

Fig 2 Sections from duodenum Tolouidine blue pH 0 5 130 × a) Control b) Rat given repeated injections of 48 80 Mast cells appear increased after 48 80 treatment

shivered and exhibited cramps of the extremities. They started to recover after about 10 minutes and appeared normal at the time of sacrifice. No gross abnormalities were found at autopsy. In particular, the gastrointestinal tract appeared normal without signs of hyperaemia or bleedings.

Skin and ears from the 48/80-treated rats exhibited a drastic reduction of mast cells, as described by *Riley* (1959). Only a few, very weakly stained mast cells with scanty granulation could be perceived per section. Sections from the control rats, on the other hand, contained a large quantity of mast cells with densely packed and heavily stained granules (Fig. 1). The mast cells of the capsular connective tissue of the thymus were greatly reduced by 48/80, as were the peribronchial and perivascular mast cells of the lungs. The same was the case with the mast cells occurring in the serous and subserous tissues in all examined organs. Mast cells of the oesophagus were similarly greatly reduced by 48/80. The submucous coat of the oesophagus normally contained 20–50 mast cells per cross section. In sections from the 48/80-treated animals, on the other hand, only a few, very weakly stained mast cells or none at all were found. A similar reaction was noted in the submucous coat of the forestomach.

Surprisingly enough, the specific type of mast cell occurring in the gastrointestinal mucosa reacted in the opposite way. These cells had the same morphological appearance and staining properties in the 48/80-treated rats as in controls. Their quantity was at any rate not reduced and in the small bowel mucosa, where the cells normally are most numerous, the 48/80 treatment actually seemed to have increased their quantity (Fig. 2). Mast cells were therefore counted and the results appear in Fig. 6 where the mast cell quantities of skin and ears have been included for comparison.

Clearly, 48/80 increased the number of mast cells per unit area of intestinal mucosa, unlike in ears and skin where their numbers were greatly reduced. The differences is significant for each separate part of the intestine, the calculated P values being: duodenum < 0.01, jejunum $= 0.02$, ileum < 0.01 and colon < 0.05. When the data from the different parts of the intestinal canal are treated according to the ranking test for grouped data the observed difference appears highly significant, P being less than 0.01.

TABLE 5

	Controls			48/80 treatment		
	Mean	Range		Mean	Range	
No. of mast cells/0.019 mm²	1 8	0 9	2 8	3 [illegible]	3 1	4 2
Nuclear diameters, scale units d	47	43	49	46	46	4[illegible]
D	78	76	81	79	[illegible]	82
Mucosal area, mm²	7 2	5 6	8 3	7 [illegible]	5 8	9 0



Figs 3–5

g 3 Mesentery spreads Toluidine blue pH 4 130 × a) Control, b) Rat given a single intravenous injection of 0 8 mg 48 80 Note the degranulation of peri capillary mast cells

g 4 Tongue sections Toluidine blue pH 0 5 330 × a) Control b) Rat given a single intravenous injection of 0 8 mg 48 80 The mast cells are degranulated

g 5 Sections from duodenum Toluidine blue pH 0 5 530 × a) Control b) Rat given a single injection of 0 8 mg 48/80 Morphological appearance and staining properties of mast cells are similar

The data recorded in Table 1 support the assumption that the increase in mast cell quantity observed reflects a real increase in cell numbers per unit volume of tissue The number of mast cells with visible nuclei per high power field of duodenal mucosa is about twice as high in 48 80 treated rats as in controls ($P = 0.05$) but the nuclear diameters are apparently unchanged The mucosal cross section areas are similar in 48 80 treated rats and in control rats which makes it very unlikely that the observed increase in mast cell numbers could be due merely to contraction of the tissue surrounding the mast cells

Acute Experiment

The rats injected with 0 2 mg of 48 80 intravenously reacted within 1–2 minutes, exhibiting signs of shock cyanosis and respiratory distress They had not shown any signs of improvement when they were killed after 15 minutes (But other rats similarly treated recovered within another hour) Rats injected with 0 8 mg reacted immediately

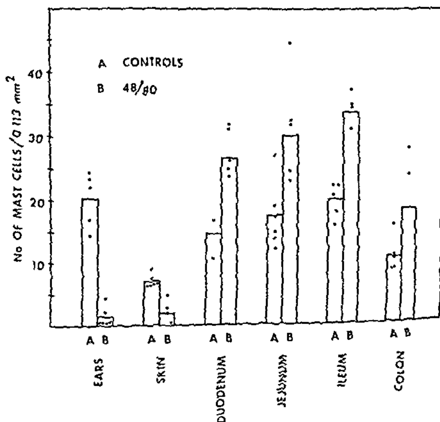


Fig 6

Mast cell counts after repeated intraperitoneal injections of 48 80 in increasing doses for 5 days Bars represent means, dots individual rats

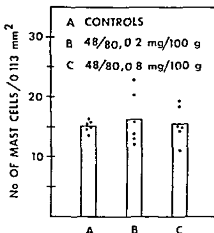


Fig 7

Mast cell counts in duodenal mucosa following single intravenous injections of compound 48 80 Bars represent means dots individual rats

after the injection, exhibited cramps of the extremities and died after 20 seconds to 2½ minutes Autopsy revealed no gross abnormalities

In the mesenteries widespread degranulation of mast cells was noted The mast cells surrounding the capillaries in the mesenterial windows seemed ruptured and their granules scattered around The reaction was only partial, however, for at a distance from the capillaries a large proportion of the mast cells appeared normal (Fig 3) No obvious differences were noted between preparations from rats treated with the two dosages of 48 80 The sections from tongue and skin displayed similar mast cell reactions in the 48 80-treated rats Again there was no obvious difference between the two dosages Practically all mast cells at both sites exhibited a very characteristic reaction similar to that noted in the mesenteries The cells were disrupted and the granules spread around the cells In addition the granules stained more weakly and in a more reddish hue than those of the controls (Fig 4) Similar reactions were not noted in any mast cells of the control rats Here a few mast cells appeared artifactively ruptured but this reaction, due to mechanical damage to the tissues during the preparation, could be easily *distinguished from that induced by 48 80*

Unlike mast cells of other tissues, those of the duodenal mucosa appeared unchanged after 48 80 Thus their morphological appearance and staining properties were the same in 48 80 treated rats as in control rats In addition the mast cell numbers per visual field of mucosa were similar in treated rats and control rats, (Fig 5 and 7)

The data recorded in Table 1 support the assumption that the increase in mast cell quantity observed reflects a real increase in cell numbers per unit volume of tissue The number of mast cells with visible nuclei per high power field of duodenal mucosa is about twice as high in 48/80-treated rats as in controls ($P = 0.05$) but the nuclear diameters are apparently unchanged The mucosal cross-section areas are similar in 48/80-treated rats and in control rats, which makes it very unlikely that the observed increase in mast cell numbers could be due merely to contraction of the tissue surrounding the mast cells

Acute Experiment

The rats injected with 0 2 mg of 48 80 intravenously reacted within 1–2 minutes, exhibiting signs of shock, cyanosis and respiratory distress They had not shown any signs of improvement when they were killed after 15 minutes (But other rats similarly treated recovered within another hour) Rats injected with 0 8 mg reacted immediately

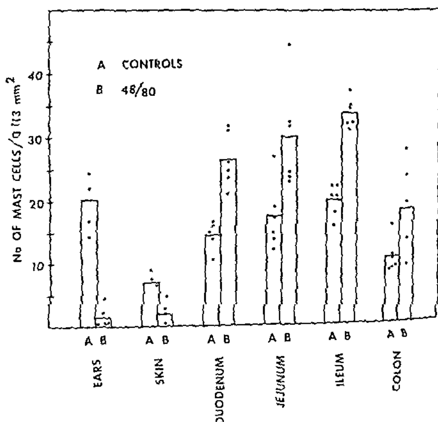


Fig 6

Mast cell counts after repeated intraperitoneal injections of 48 80 in increasing doses for 5 days Bars represent means dots individual rats

The present communication has clearly demonstrated that the specific type of mast cell occurring in large quantities in the intestinal mucosa differs from other mast cells in being insensitive to the degranulating effect of Compound 48 80 Further studies on the chemical organization of the two types of cell in particular their enzyme content could give additional information on the mechanism of the degranulation process

The surprising finding of an almost twofold increase in intestinal mast cells after repeated injections of 48 80 when other tissues are depleted of mast cells at present lacks a satisfactory explanation More experimental work in particular a study of the long term effect of Compound 48 80 will be necessary before this finding can be evaluated The apparent increase of mast cells in intestinal mucosa could be either a direct or an indirect effect of 48.80 related to its degranulating or destructive action on other mast cells The latter possibility appears the most plausible but this could be clarified by studying the effect on intestinal mast cells of other mast cell degranulating substances such as polymyxin B The problem deserves further study for very little is actually known about factors regulating the formation of mast cells in tissues

The histamine in most tissues is probably bound to mast cells but the histamine in the gastrointestinal mucosa has been considered one of the exceptions to this rule Some of the evidence supporting this opinion is interesting in connection with the findings reported here In the case of the rat this assumption was based on the inability to demonstrate significant quantities of mast cells in gastrointestinal mucosa (*Mota et al* 1956 *Riley* 1959) and on the finding that intestinal histamine is not reduced following 48 80 treatment (*Mota et al* 1956 *Riley* 1959) The susceptibility of intestinal mast cell granules to common fixatives (*Enerback* 1966) and the differences in dye binding properties between intestinal and other mast cells in the rat (*Enerback* 1966a) are probably responsible for the difficulties in demonstrating these cells Since the intestinal mast cells are insensitive to the degranulating effect of Compound 48 80 the failure of this drug to deplete intestine of its histamine content could be taken as an argument for rather than against intestinal histamine being of mast cell origin

SUMMARY

The mast cells occurring in large quantities in the gastrointestinal mucosa have been reported to differ from other mast cells in the rat both histochemically and morphologically The present communication describes the reactivity of these cells towards the histamine liberator Compound 48 80 Intravenous injection of 0 2 and 0 8 mg 48 80 per 100g resulted in degranulation of the mast cells in mesentery tongue and skin Mast cells in duodenal mucosa on the other hand appeared

unaffected Intraperitoneal injections of 48/80 in increasing doses twice daily for 5 days resulted in almost complete disappearance of mast cells from all examined tissues except the gastrointestinal mucosa On the contrary, counts in different parts of the intestinal mucosa revealed an almost twofold increase in mast cells

REFERENCES

Baltzly R Buck J S De Beer E J & Webb F J A family of long acting depressors J Am Chem Soc *71* 1301 1305 1949

Benditt E P & Arase M An enzyme in mast cells with properties like chymotrypsin J Exp Med *110* 451–460 1959

Boreus L O A comparison of mast cell reactions in the rat hamster and guinea pig Acta Physiol Scand *49* 251 260 1960

Enerbäck L Mast cells in rat gastrointestinal mucosa 1 Effects of fixation Acta path et microbiol scandinav *66* 289–302 1966

Enerbäck L Mast cells in rat gastrointestinal mucosa 2 Dye binding and metachromatic properties Acta path et microbiol scandinav *66* 303 312 1966

Fawcett D W Cytological and pharmacological observations on the release of histamine by mast cells J Exp Med *100* 217 224 1954

Högberg B & Uvnäs B The mechanism of the disruption of mast cells produced by compound 48/80 Acta Physiol Scand *41* 345–369 1957

Lagunoff D & Benditt E P A new method for study in vitro of morphologic and chemical changes in mast cells J Histochem Cytochem *7* 286 1959

Lagunoff D & Benditt E P Mast cell degranulation and histamine release observed in a new in vitro system J Exp Med *112* 571 580 1960

Lagunoff D & Benditt E P Proteolytic enzymes of mast cells In Mast cells and basophils Padawer J Ed Ann N Y Acad Sci *103* 1–492 1963

Mota I Beraldo W T & Junqueira L C U Protamine like property of compounds 48/80 and Stilbamidine and their action on mast cells Proc Soc Exp Biol (N Y) 83 455 457 1953

Mota I Beraldo W Ferri A G & Junqueira L C U Action of 48/80 on the mast cell population and histamine content of the wall of the gastrointestinal tract of the rat Ciba Foundation Symposium on Histamine J & A Churchill Ltd London pp 47 50 1956

Mota I Ferri A G & Yoneda S The distribution of mast cells in the digestive tract of laboratory animals Its bearings on the problem of the location of histamine in tissues Quart J micr Sci *97* 251 256 1956

Mota I & Vugman I Effects of anaphylactic shock and compound 48/80 on the mast cells of the guinea pig lung Nature (Lond) *177* 427–429 1956

Mota I & Vugman I Action of compound 48/80 on the mast cells and histamine content of guinea pig tissues Brit J Pharmacol *11* 304 307 1956 a

Norton S Quantitative determination of mast cell fragmentation by compound 48/80 Brit J Pharmacol *9* 494–497 1954

Paton W D M Compound 48/80 A potent histamine liberator Brit J Pharmacol 6 499 508 1951

Paton W D M & Schachter M The influence of an antihistaminic drug on the release of histamine in the unanaesthetized dog Brit J Pharmac 1 6 509 513 1951

Riley J F The mast cells E & S Livingstone Ltd Edinburgh and London 1959

Riley J F & West G B The presence of histamine in tissue mast cells J Physiol (Lond *120* 528–537 1953

Riley J F & West G B Tissue mast cells Studies with a histamine liberator of low toxicity (compound 48/80) J Path Bact *69* 269 282 1955

Riley J F & West G B Histamine liberation in the rat and mouse Arch Int Pharmacodyn *102* 304 313 1955 a

Uvnäs B & Thon I L Isolation of biologically intact mast cells Exp Cell Res *18* 512 520 1959

Wilcoxon F Probability tables for individual comparisons by ranking methods Biometrics 3 119 122 1947

Acta path et microbiol scandinav 66 323 326 1966

The Department of Forensic Medicine (Head Prof J Raekallio M D) and
The Department of Pathological Anatomy (Head Prof Osmo Jarvi, M D),
University of Turku Turku Finland

ACCUMULATION OF TETRACYCLINES IN ATHEROSCLEROTIC LESIONS OF HUMAN AORTA

By

I Lindgren and J Raekallio

Received 6 ix 65

In human atherosclerosis, accumulation of acid mucopolysaccharides precedes the depositing of lipids and, later on, of calcium salts (*Lorenzen* 1963) Especially large amounts of acid mucopolysaccharides are noted in cases of idiopathic medionecrosis of aorta (*Raekallio* 1958) Tetracyclines have an affinity to human atherosclerotic lesions (*Malek* 1962), also *in vitro* (*Levonen & Mustakallio* 1964) In rats tetracyclines are deposited in arterial walls during parathyroid hormone overdosage

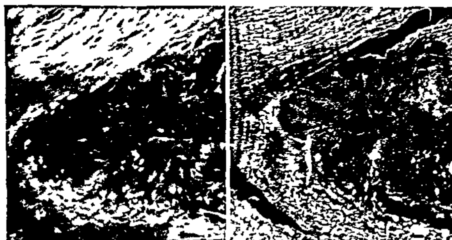


Fig 1 *Fig 2*

Figs 1 and 2

Aorta from an individual who had received tetracycline prior to death Fig 1 to the left is a fluorogarm of an unstained transversal section showing tetracycline deposits along the border of an atherosclerotic intimal plaque to the right of the center Fig 2 is a microradiogram of an adjacent section The bright spots are mineral deposits located in the same areas as tetracyclines in Fig 1 However there were minerals but no tetracyclines in the middle of the plaque Magnification 200 ×

This work was supported by grants from the *Sigrid Juselius Foundation* and from the *Finnish Medical Council*

(*Häkkinen & Lindgren* 1963) In the present work, therefore, an attempt has been made to correlate the localization of tetracyclines in human atherosclerotic lesions to changes demonstrable both histochemically and histophysically

MATERIAL AND METHODS

Autopsy specimens of human aortas showing atherosclerotic changes of varying severity were collected. The causes of death varied but all the patients had been treated with 2 12 g of tetracyclines during the last days. Paraffin sections after neutral formalin were used as well as frozen sections

Tetracyclines were observed in untreated sections by fluorescence microscopy with an Osram high vapour mercury lamp HBO and filters B 12 and OG 1 The wavelength of the transmitted light was 550 Å The corresponding sections were then studied by microradiography at a wavelength of 1 5 Å (*Engström* 1956) for mineral deposits and dry weight, by the Alcian blue method (*Lison* 1954) for acid mucopolysaccharides and by the Oil red isopropylalcohol technique (*Lillie* 1944) for lipids In addition the sections were investigated histologically



Fig 3



Fig 4

Figs 3 and 4

Specimen as in Fig 1 Fig 3 to the left is a fluorogram of an unstained section of an intimal plaque Media is at the bottom. The bright spots in Fig 3 are due to tetracycline deposits. Fig 4 is a photomicrogram of the same section stained with Alcian blue The Alcian blue reaction, although faintly reproducible in black white can be correlated to tetracycline fluorescence Magnification 200



Fig 5 *Fig 6*

Figs 5 and 6

ch
of
of

RESULTS

Comparison of fluorograms and microradiograms revealed that tetra cyclines deposited mainly in the same areas as mineral salts However there were areas which contained mineral salts but no tetracyclines for example the middle avascular part of the plaque (Figs 1 and 2) Acid mucopolysaccharides were correlated well to the tetracycline deposits However the tetracycline fluorescence could be more easily detected than the Alcian blue reaction and there were areas which showed weak or no Alcian blue staining but did contain remarkable amounts of tetracycline (Figs 3 and 4) Lipid deposits and tetracycline fluorescence showed no distinct correlation the areas containing

large amounts of lipids took up only small amounts of tetracyclines (Figs 5 and 6)

COMMENT

Tetracyclines have an affinity to sites where calcium is known to accumulate (*Malek* 1962) Indeed, we observed tetracycline fluorescence mostly in areas containing mineral salts and/or acid mucopolysaccharides The latter may bind calcium ions (*Boyd & Neuman* 1951) There was some additional tetracycline fluorescence in the areas containing mucopolysaccharides, lipids or minerals Tetracyclines were seen to have an affinity to necrobiotic sites, too, even before the imminent necrosis was recognizable histochemically (*Kovacs et al* 1964) Thus tetracycline fluorescence in the aorta, among other things may be one of the earliest indicators of atherosclerotic lesions

SUMMARY

With the aid of fluorescence microscopy, microradiography and histochemical methods, accumulation of tetracyclines was studied in atherosclerotic lesions of human aortas The cause of death varied but all the patients had been treated with 2 12 g of tetracyclines during the last days Tetracycline fluorescence was observed in areas containing mineral salts and/or acid mucopolysaccharides In addition, tetracyclines accumulated in necrobiotic sites

REFERENCES

... The surface chemistry of bone ion binding properties

hysical techniques in
p 489 1956
the metastatic calci
... terol

technic Stain Technol *19* 55 1944

Lison L Alcian Blue 8 G with chlorantine Fast Red 5 B A technic for selective staining of mucopolysaccharides Stain Technol *29* 131 1954

Loren en I Experimental arteriosclerosis Munksgaard Copenhagen 1963

Malek P Tetracyklinova antibiotika v organismus Československá Akademie Věd Praha 1962

Raekallio J Histochemical studies on idiopathic medionecrosis of the aorta A M A Arch Path 66 733 1958

Acta path et microbiol scandinav 66 327–330 1966

The Department of Pathology, Section II University of Helsinki and from the District Hospital of Hyvinkaa Hyvinkaä, Finland

RAMIFYING AND ADHESIVE MUCOSAL FOLDS OF THE GALL BLADDER

A Cause of Distress?

By

H TEIR, G ELFVING and T LEHTONEN

Received 8 ix 65

It is sometimes necessary with gall bladder complaints to resort to surgical management of patients whose gall bladders can be seen roentgenologically to fill poorly and or contract poorly The exposed gall bladder is, however, fairly normal in appearance or resembles at the most slight cholesterolosis Routine histologic examination shows hardly any pathologic changes in these cases

We have paid special attention to these cases in routine study of excised gall bladders and tried histologically to find common features in them They surprinsingly often showed considerable abnormal folds which may be associated with the symptoms This is a preliminary report on our observations We shall later publish a more detailed report in which special attention will be devoted to structural changes and to the pre- and postoperative clinical picture of these patients

MATERIAL AND METHODS

The material consisted of 871 patients with gall bladder disease operated on since 1960 at the District Hospital of Hyvinkaa Cases with gallstones tumour and manifest inflammation were excluded from the series and also cases in which cholesterolosis was demonstrated microscopically There thus remained 53 patients whose gall bladders appeared to be fairly normal both grossly and microscopically using the customary criteria Two of these cases displayed no abnormal changes The folds was also shallow structurally

The excised gall bladder was cut in the longitudinal direction in its entirety A specimen measuring about 2 × 2 cm was cut with scissors for histologic study always from the same site in the anterior part of the gall bladder so that a part of the fundus was included in the specimen If changes could be established at the macroscopic examination a sample was taken also from these sites

Special attention was paid to the structure of the gall bladder mucosa in the histologic examination Long and ramifying irregular and asymmetrical folds were [illegible]

of all the other layers together

Thirty of the patients were women 21 were men Their age range was 7 69 years



Figs 1–2

Fig 1 Grade I. 21-years old woman with 5 years of pain on the right in the epigastrium Symptom-free after operation (× 50)

Fig 2. Grade I 27-years-old woman with 7 years of pain and dyspepsia Slight dyspepsia after operation (× 50)

RESULTS

Ramifying and adhesive folds were observed in 51 patients of whom 28 had changes of group 1 (Figs 1–2) and 23 of group 2 (Figs. 3–5) All the patients had dyspeptic complaints All but one stated that they had had some kind of pain But severe episodes of pain were not



Figs 3–4

Fig 3 Grade II 7-year-old girl, who's short history is given in the text (× 50)

Fig 4 Grade II 39-year old man with over 10 years of biliary symptoms Symptom-free after operation (× 50)

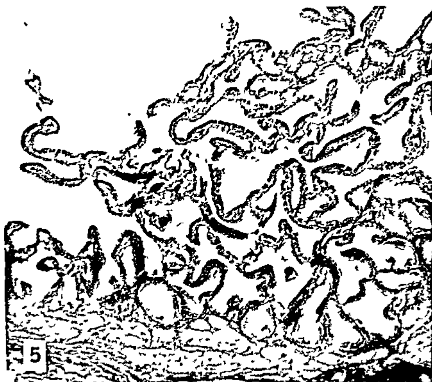


Fig 5
Grade II 42 years old man with 2 years of pains and dyspepsia
Symptom free after operation (× 50)

reported on the whole The duration of the symptoms had ranged from 1 month to over 10 years Thirtysix of the pictures of the patients given a roentgenigraphic examination were available for study The gall bladders in all of them filled poorly and/or contracted poorly

A follow-up examination was performed on all 51 patients an average of 2 5 (1 month—5 years) years after cholecystectomy All but one were free of their pain In both groups over 75 per cent of the patients were free of all dyspeptic distress as well As an example may be mentioned a girl aged 7 who had a history of over 2 5 years of severe dyspeptic complaints and attacks of pain with vomiting (Fig 3) She was able immediately postoperatively to take any kind of food and has been free of symptoms for 3 years now

DISCUSSION

The results of the investigation suggest that there is a correlation between clinical complaints and fold hyperplasia Similar changes appear in connection with cholelithiasis and cholesterolosis, but that is

a problem outside the scope of the present work although it may give symptoms identical to those of described here

We have not found any observations to this effect in the literature The reason may be that adhesive fold hyperplasia is regarded as a banal and secondary change Its etiology and pathogenesis are still unclarified It is understandable, especially in the cases of group 2, that the normal function and motility of the gall bladder are disturbed, and this can be established roentgenologically More accurate clarification of the disease will depend on further studies in connection with which an endeavour will also be made to establish the foundary line between normal and hyperplastic folds

Acta path et microbiol scand vol 66 331–340 1966

The University of Bergen School of Medicine the Gade Institute
Department of Pathology Bergen Norway (Head Erik Waaler M D)

ATHEROSCLEROSIS IN AN AUTOPSY SERIES

7 *Relation of Hypertension to Atherosclerosis*

By

J CHR GIERTSEN

Received 8 x 65

In all the reports submitted to the Deuxieme Conference Internationale de Pathologie Geographique and in which the relation of the blood pressure to atherosclerosis had been considered *Anitschkow* (2) found a higher incidence of atherosclerosis in hypertensive than in normotensive individuals The results of most other investigations are in accordance with this (*Ackerman Dry & Edwards* (1) *Baker & Iannone* (3) *Baker Iannone & Kinnard* (4) *Clawson* (6) *Creed Baird & Fischer* (7) *Davis & Klainer* (9) *Lober* (15) *Parrish* (17) *Wilkins Roberts Jr & Moses* (20) *Winter Jr Sayre Millikan & Barker* (21)) There are naturally a few variations in the findings particularly with regard to the age at which the effect of hypertension is most pronounced For instance Anitschkow found the effect to be most pronounced in individuals younger than 60 years in the Zurich series but in individuals older than 50 years in the Vienna series whereas *Ackerman Dry & Edwards* and *Baker & Iannone* found an effect in all age groups

Mitchell Schwartz & Zinger (16) however found an effect only on the area of raised plaques not on the area of fatty streaking and *Bjornsson* s (5) and *White Edwards & Dry* s (19) series failed to show any effect at all of hypertension

Davis & Klainer (10) contended that hypertension per se was not the cause of the higher incidence of atherosclerosis in hypertensive individuals but that the two conditions have a common aetiological factor Nor did *Holman & Moossy* (14) attach any great significance to hypertension when they stated that less than a quarter (and probably less than 10 %) of the lesions of atherosclerosis can be accounted for on a basis of hypertension hypercholesterolemia or obesity On the other hand *Paterson Mills & Lockwood* (18) maintain that once the atherosclerotic lesion has been formed hypertension—transient or persistent—is a most important accelerating factor in the further development of the process

MATERIAL

The material has been previously described (*Giertsen* (12 13)) It consists of 408 individuals 211 males and 197 females ranging in age from 15 to 89 years The mean age was 62 3 years in males and 62 9 in females The total cholesterol/phospholipid ratio in the arterial wall—the ChPh value—has been used as index for the severity of atherosclerosis (*Giertsen* (12))

The series has been divided into two main groups of normotensive and hypertensive individuals From the latter group individuals with cardiac hypertrophy were singled out and examined as a sub group

The blood pressure readings in the clinical records were used for the classification of normotension and hypertension In some instances only the admission reading was available but in most cases several readings had been made The criteria for normal and abnormal blood pressure endorsed by the American Heart Association's Conference on the Epidemiology of Cardiovascular Diseases (11) have been applied in the classification The series was too small to warrant a distinction between the various types of hypertension namely borderline hypertension systolic or diastolic hypertension only and both systolic and diastolic hypertension Consequently, all individuals with a blood pressure below 140/90 were classified as normotensive and those with a higher pressure as hypertensive No distinction was made between essential and renal hypertension

The heart weight was classified according to Zeek's (23) tables A heart was considered to be hypertrophic when its weight exceeded the normal weight for the individual's height plus twice the standard deviation

As will be seen in subsequent papers individuals with malignant disease may have less atherosclerosis and individuals with atherosclerotic heart disease have more atherosclerosis than others Therefore the series has been cleared of individuals with these two conditions and the relation of blood pressure to atherosclerosis has been examined in the remaining series

The normotensive and hypertensive groups were sub divided into age and sex groups For each group the mean ChPh value has been calculated and for groups of 6 or more cases also the standard error of the mean

RESULTS

Table 1 shows the age and sex distribution of the normotensive and hypertensive individuals in the total series and in the cleared series It appears that in the total series there is an equal number of normotensive and hypertensive males but considerably more hypertensive than normotensive females Slightly more than half of the hypertensive individuals had cardiac hypertrophy Below 40 years of age the groups are generally small In the cleared series there are more hypertensive than normotensive individuals and many groups are poorly represented

Table 2 and Fig 1 show the results in the total series With the exception of a few age groups hypertensive individuals have more atherosclerosis than normotensive especially when cardiac hypertrophy is also present In the coronary arteries the effect of hypertension is very pronounced in the youngest age-groups Thereafter the effect diminishes with age, and disappears in the oldest age groups In the aorta the effect is not equally pronounced in the younger age groups but again there is a tendency of the effect to diminish with age In the cerebral arteries the effect is just as pronounced in old as in young age and after 40 years of age the curves for hypertensive and normotensive individuals are almost parallel Only in hypertensive individuals does cerebral atherosclerosis reach approximately the same severity that

aortic and coronary atherosclerosis may reach in normotensive individuals Obviously, hypertension does not affect the atherosclerotic process at a proportionally equal rate in these three arteries

Table 3 and Fig 2 show that the results are essentially the same when the series is cleared of individuals with malignant disease and athero sclerotic heart disease

There is no obvious difference between males and females in the effect of hypertension

The individual variation of the ChPh value within the groups is wide, and the standard error of the mean is fairly high

TABLE 1

The Age and Sex Distribution of Individuals with Normotension and Hypertension and of Individuals with Hypertension and Cardiac Hypertrophy (CH)

Age group	*Males*			*Females*		
	Normo tension	Hypertension		Normo tension	Hypertension	
		Total number	With CH		Total number	With CH
			Total series			
15–19	3					
20 29	4			3	2	
30–39	8	9	4	17	2	2
40 49	10	9	8	7	10	4
50–59	19	18	12	10	21	8
60–69	27	30	18	17	31	15
70–79	21	28	15	20	33	23
80–89	14	11	7	7	17	9
Total	106	105	64	81	116	61
		Individuals with malignant disease and atherosclerotic heart disease excluded				
15 19	2					
20 29	3			2	2	
30 39	4	5	4	9	2	2
40–49	3	7	7	3	6	3
50 59	9	11	7	4	13	5
60–69	8	13	7	11	16	8
70–79	8	13	9	7	15	9
80–89	7	6	4	3	7	3
Total	44	55	38	39	61	30

DISCUSSION

A post mortem classification of a clinical condition like hypertension is not without certain pitfalls Two methods are available namely classification according to the heart weight at autopsy and according to the actual blood pressure readings in the clinical records Both methods present advantages and disadvantages When the former is

MATERIAL

The material has been previously described (*Giertsen* (12 13)) It consists of 408 individuals 211 males and 197 females ranging in age from 15 to 89 years The mean age was 62 3 years in males and 62 9 in females The total cholesterol phospholipoid ratio in the arterial wall—the ChPh value—has been used as index for the severity of atherosclerosis (*Giertsen* (12))

The series has been divided into two main groups of normotensive and hypertensive individuals From the latter group individuals with cardiac hypertrophy were singled out and examined as a sub group

The blood pressure readings in the clinical records were used for the classification of normotension and hypertension In some instances only the admission reading was available but in most cases several readings had been made The criteria for normal and abnormal blood pressure endorsed by the American Heart Association s

essential and renal hypertension

The heart weight was classified according to *Zeek* s (23) tables A heart was considered to be hypertrophic when its weight exceeded the normal weight for the individual s height plus twice the standard deviation

As will be seen in subsequent papers individuals with malignant disease may have less atherosclerosis and individuals with atherosclerotic heart disease have more atherosclerosis than others Therefore the series has been cleared of individuals with these two conditions and the relation of blood pressure to atherosclerosis has been examined in the remaining series

The normotensive and hypertensive groups were sub divided into age and sex groups For each group the mean ChPh value has been calculated and for groups of 6 or more cases also the standard error of the mean

RESULTS

Table 1 shows the age and sex distribution of the normotensive and hypertensive individuals in the total series and in the cleared series It appears that in the total series there is an equal number of normotensive and hypertensive males, but considerably more hypertensive than normotensive females Slightly more than half of the hypertensive individuals had cardiac hypertrophy Below 40 years of age the groups are generally small In the cleared series there are more hypertensive than normotensive individuals, and many groups are poorly represented

Table 2 and Fig 1 show the results in the total series With the exception of a few age-groups, hypertensive individuals have more atherosclerosis than normotensive, especially when cardiac hypertrophy is also present In the coronary arteries the effect of hypertension is very pronounced in the youngest age-groups Thereafter, the effect diminishes with age, and disappears in the oldest age-groups In the aorta the effect is not equally pronounced in the younger age-groups, but again there is a tendency of the effect to diminish with age In the cerebral arteries the effect is just as pronounced in old as in young age and after 40 years of age the curves for hypertensive and normotensive individuals are almost parallel Only in hypertensive individuals does cerebral atherosclerosis reach approximately the same severity that

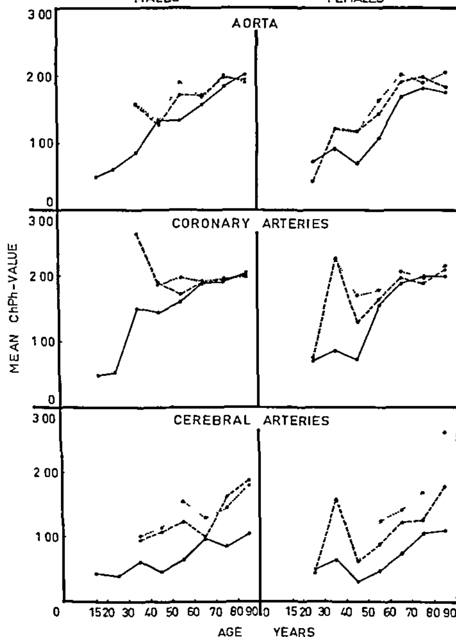


Fig 2

The variation with age of the mean aortic coronary and cerebral ChPh value in normotensive and hypertensive males and females Individuals with malignant *disease and atheroscler 1 c heart disease excluded*

tensive individual is classified as normotensive or vice versa, all such erroneous classifications will lead in only one direction, *they will tend to minimize the effect of hypertension* Any effect which is found will *therefore be a minimum effect*

In spite of the classification difficulties a definite effect of hypertension on the atherosclerotic process has been found in this series, as *in most others It seems only reasonable that the effect should be most* pronounced in individuals in whom the hypertension has been especially severe or of long standing, as evidenced by the cardiac hypertrophy *The diminishing effect with age was also found in the coronary arteries by I ober (15), who noted a flattened curve for hypertensive individuals and this was due to the fact that hypertensive hearts showed maximum measurable degree of atherosclerosis* in almst all age groups This is not, however, the fact in the aorta and the cerebral arteries In these the *effect is not as pronounced, in the younger age groups, as in the coronary arteries In addition, the effect is very marked also in old age in the* cerebral arteries This is in agreement with *Baker & Iannone*'s (3) findings Thus, it seems that these arteries have a "reserve capacity", not possessed by the aorta and the coronary arteries, to develop more atherosclerosis in old age when hypertension occurs, and not till then *do the cerebral arteries develop the severe degree of atherosclerosis that may be found in the aorta and the coronary arteries in normo*tensive individuals The disproportionate effect of hypertension on aortic, coronary, and cerebral athersclerosis may possibly be due to a *modifying effect of local factors Holman & Moossy (14) for instance* pointed out that the cerebral arteries present special anatomical and physiological characteristics which may be of importance in the pathogenesis of atherosclerosis in these arteries

In a previous paper (*Giertsen* (13)) it was stressed that if a factor does influence the progress of atherosclerosis but not at a proportionally equal rate in all arteries, the results of a study of that factor's influence would depend on which artery is examined Obviously hypertension is such a factor, and conclusions from a study of one artery are not valid for another artery without reservation *The disproportionate effect of hypertension may also in part explain the great individual variations which were found in the inter relationship between* atherosclerosis in the aorta, the coronary, and the cerebral arteries

It would obviously be of interest to study the correlation between the ChPh value and the height of the systolic and diastolic blood pressure within each age and sex group but the present series was too small to permit such a study

SUMMARY

The relation of hypertension to atherosclerosis in the aorta, the coronary, and the cerebral arteries has been examined in 408 individuals, 211 males, and 197 females, ranging in age from 15 to 89 years

The total cholesterol phospholipoid ratio in the arterial wall the ChPh-value—has been used as index for the severity of atherosclerosis

The blood pressure was classified according to the actual blood pressure readings in the clinical records The series was divided into two groups, normotensive and hypertensive individuals From the latter group individuals who also had cardiac hypertrophy were singled out and studied separately The series was then cleared of individuals with malignant disease and atherosclerotic heart disease and examined in a similar way

Hypertensive individuals, especially those with cardiac hypertrophy, had more atherosclerosis than normotensives in most age groups The effect of hypertension was most pronounced in the younger age-groups in the coronary arteries In the aorta and the coronary arteries, the effect diminished or disappeared completely with age, but in the cerebral arteries it was marked also in old age This disproportionate effect of hypertension in the three arteries shows that conclusions from a study of the effect of hypertension on atherosclerosis in one artery cannot be valid for another artery It may also in part explain the great individual variations which have been previously found in the inter relationship between atherosclerosis in the aorta, the coronary, and the cerebral arteries

The cleared series showed essentially the same findings The effect of hypertension did not show any obvious sex difference

REFERENCES

1 *Ackerman R F Dry T J & Edwards J F* Relationship of various factors to the degree of coronary atherosclerosis in women Circulation *1* 1345–1354 1950

2 *Anitschkow N* Pathologische Anatomie und allgemeine Pathologie der Arterio sklerose Comptes Rendus de la Deuxieme Conference Internationale de Pathologie Géographique Utrecht 1934 pp 44 101

3 *Baker A B & Iannone A* Cerebrovascular disease VII A study of etiologic mechanisms Neurology 11 (Part 2) 23 31 1961

4 *Baker A B Iannone A & Kinnard J* Cerebrovascular disease VI Relationship to disease of the heart and the aorta Neurology *11* 63 70 1961

5 *Bjornsson J* Arteriosclerosis Munksgaard Copenhagen 1942

6 *Clawson B J* Coronary sclerosis An analysis of nine hundred twenty-eight cases Am Heart J *17* 387 400 1939

7 *Creed D L Baird W F & Fischer F R* The severity of aortic arteriosclerosis in certain diseases A necropsy study Am J M Sc *230* 385–391 1955

8 *Davis D & Blumgart H L* Cardiac hypertrophy relation to coronary arterio sclerosis and congestive heart failure Ann Int Med *11* 1024–1038 1937

9 *D* [illegible]

10 *D* [illegible]

11 Epidemiology of Cardiovascular diseases Methodology Supplement to Am J Pub Health *50* 1 38 1960

12 *Giertsen J Chr* Atherosclerosis in an autopsy series 1 Chemical grading of atherosclerosis Acta path et microbiol scandinav Supplementum *170* 1964

13 *Giertsen J Chr* Atherosclerosis in an autopsy series 3 Inter relationship between atherosclerosis in the aorta the coronary and the cerebral arteries Acta path et microbiol scandinav 63 391 413 1965

14 *Holman R L & Moossy J* The natural history of aortic coronary and cerebral atherosclerosis In Fields W S (Ed) Pathogenesis and treatment of cerebro vascular disease Charles C Thomas Springfield Ill 1961 pp 39 54

15 *Lober P H* Pathogenesis of coronary sclerosis Arch Path 55 357–383 1953

16 *Mitchell J R A Schwartz C J & Zinger A* Relationship between aortic plaques and age sex and blood pressure Brit Med J 1 205 209 1964

17 *Parrish H M* Epidemiology of ischemic heart disease among white males II Autopsy incidence of ischemic heart disease and autopsy prevalence of coronary atherosclerosis J Chronic Dis 14 326–338 1961

18 *Paterson J C Mills J & Lockwood C H* The role of hypertension in the progression of atherosclerosis Canad M A J 82 65–70 1960

19 *White N K Edwards J E & Dry T J* The relationship of the degree of coronary atherosclerosis with age in men Circulation 1 645 654 1950

20 *Wilkins R H Roberts Jr J C & Moses C* Autopsy studies in atherosclerosis III Distribution and severity of atherosclerosis in the presence of obesity hypertension nephrosclerosis and rheumatic heart disease Circulation 20 527 536 1959

21 *Winter Jr W D Sayre G P Millikan C H & Barker N W* Relationship of degree of atherosclerosis of internal carotid system in the brain of women to age and coronary atherosclerosis Circulation 18 7–18, 1958

22 *Yater W M Welsh P P Stapleton J F & Clark M L* Comparison of clinical and pathologic aspects of coronary artery disease in men of various age groups A study of 950 autopsied cases from the Armed Forces Institute of Pathology Ann Int Med 34 352 392 1951

3 *Zeek P M* Heart weight. I The weight of the normal human heart Arch Path 34 820 832 1942

Acta path et microbiol scandinav 66 341 350 1966

The University of Bergen School of Medicine, the Gade Institute
Department of Pathology, Bergen Norway (Head Erik Waaler, M D)

ATHEROSCLEROSIS IN AN AUTOPSY SERIES

8 *Relation of Malignant Disease to Atherosclerosis*

By

J CHR GIERTSEN

Received 8 x 65

A considerable number of investigations have been concerned with the inter relationship between atherosclerosis and cancer *Busch* (6) was one of the first to take up the question He found that a considerable number of cancer patients had an aorta completely devoid of lipoid infiltration and sclerosis with its consequences This was confirmed by *Casper* (8) *Anitschkow* (1) found that in all but two of the reports submitted to the Deuxieme Conference Internationale de Pathologie Géographique cancer patients showed considerably less atherosclerosis than others One of the exceptions was the Swedish series but in the separately published results of the Swedish study *Sjovall & Wihman* (32) also found less advanced atherosclerosis in cancer patients Many later investigations show similar results These studies have mainly been concerned with malignant disease as one collective group (*Baker & Iannone* (2), *Baker, Kinnard & Iannone* (3), *Bähr* (7), *Dormanns & Emminger* (10), *Juhl* (24), *Lober* (26), *Schneider* (31), *Tichy* (34), *Wanscher & Clemmesen* (35)) Special types of malignant disease have also been considered separately, such as leukaemia (*Janssen, Hevelke, Mielke & Schulz* (23)), malignant lymphoma (*Creed Baird & Fischer* (9)), and multiple myeloma (*Spain, Greenblatt, Snapper & Cohn* (33))

Special attention has been paid to bronchial carcinoma *Burkhardt* (5) found that individuals with this tumour had more atherosclerosis than individuals with gastric carcinoma In *Juhl s* (24) series males with bronchial carcinoma showed more atherosclerosis than males with other types of cancer but less than non cancer males *Creed, Baird & Fischer* (9) and *Meriggi* (28) found that individuals with bronchial carcinoma had even more atherosclerosis than the non cancer control individuals On the other hand *Tichy* (34) did not find any difference in the severity of atherosclerosis between individuals with bronchial carcinoma and individuals with other malignant tumours

In a series of studies *Elkeles* (12, 13 14 15 16) found, roentgenologically, that cancer patients especially those with stomach cancer, displayed fewer calcified plaques in the aorta than a control series of

patients without cancer. Patients with cancer of the respiratory tract did not differ from the control series. This was taken as supportive evidence of a lower incidence of atherosclerosis in cancer patients. *Dungal & Benediktsson* (11) confirmed Elkeles' findings with regard to calcification in the aorta and carcinoma of the stomach in an autopsy series. It is, however, questionable whether the severity of atherosclerosis really can be measured roentgenologically. *Hyman & Epstein* (22) maintained that this could be done, but *Moran, Schwartz & Ungar* (29) could not demonstrate a good relationship between the extent of roentgenological calcification and the severity of atherosclerosis. Moreover *Winkelstein, Lilienfeld Jr., Pickren & Lilienfeld* (36) could not confirm Elkeles' findings.

Finally, it should be mentioned that *Bjornsson* (4) did not find any difference in the severity of atherosclerosis between cancer and non-cancer individuals and *Parrish* (30) denied that there is any correlation either positive or negative between the two conditions.

MATERIAL

The material has been previously described (*Giertsen* (18, 19)). It consists of 408 cases, 211 males and 197 females, ranging in age from 15 to 89 years. The mean age was 62.3 years in males and 62.9 in females. The total cholesterol/phospholipoid ratio in the arterial wall—the ChPh value—has been used as index for the severity of atherosclerosis (*Giertsen* (18)).

The series was divided into two main groups, *viz.* individuals without and with malignant disease (code numbers 140 to 205 in the Manual of the International Classification of Diseases, Injuries and Causes of Death (27)). Thereafter the series was cleared of individuals with hypertension and atherosclerotic heart disease and the remaining series examined separately. No attempt was made to study the relation of various types of malignant disease to atherosclerosis because the series was too small. There were, for instance, only 9 cases with bronchial carcinoma.

Each main group was sub-divided into age and sex groups. For each group the mean ChPh value was calculated, and for groups of 6 or more cases also the standard error of the mean.

RESULTS

The age and sex distribution of the individuals within the two main groups are given in Table 1. It appears that there is about the same number of males as females with malignant disease. When individuals with hypertension and atherosclerotic heart disease are excluded from the series, the group without malignant disease is reduced proportionately considerably more than the group with malignant disease.

Table 2 and Fig. 1 show that individuals with malignant disease have on an average a lower ChPh value than individuals without malignant disease. *The difference is present in both sexes in all age groups* except in the higher and lower.

When the series is cleared of individuals with hypertension and atherosclerotic heart disease the difference is no longer as consistent as in the total series, as shown in Table 3 and Fig. 2. In many age groups individuals with malignant disease have more atherosclerosis

than those without malignant disease In the cerebral arteries there is no longer any consistent trend

The individual variation within the groups is very wide, and the standard error of the mean is fairly high

TABLE 1

The Age and Sex Distribution of Individuals without and with Malignant Disease

Age group	*Males*		*Females*	
	Without malignant disease	With malignant disease	Without malignant disease	With malignant disease
		Total series		
15 19	2	1		
20–29	3	1	4	1
30–39	9	8	11	8
40–49	15	4	9	8
50–59	28	9	20	11
60–69	41	16	34	14
70–79	35	14	39	14
80–89	21	4	21	3
Total	154	57	138	59
	Individuals with hypertension and atherosclerotic heart disease excluded			
15–19	2	1		
20–29	3	1		
30–39	4	4	9	8
40–49	3	4	3	4
50–59	9	7	4	5
60 69	8	6	11	3
70–79	8	6	7	7
80–89	7	3	3	1
Total	44	32	39	29

DISCUSSION

The findings in the total series seem to indicate that individuals with malignant disease have less atherosclerosis than individuals without any such disease The latter group was however most heavily weighted with individuals with hypertension and atherosclerotic heart disease When the series was cleared of individuals with these two conditions the difference between the two groups was reduced and the trend was no longer quite as clear as in the total series This might suggest that the difference found in the total series is not real

The groups in the cleared series are small however and there is a great variation of the ChPh values within the groups This means that the inclusion of only a few more cases in either of the sub groups may alter the *picture considerably Possibly no definite conclusions should* be drawn from the results obtained in this series

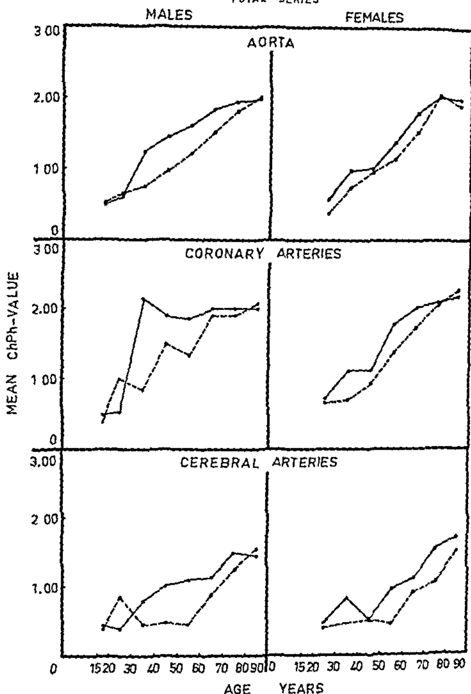


Fig 1

The variation with age of the mean aortic, coronary and cerebral ChPh value in males and females without and with malignant disease Total series

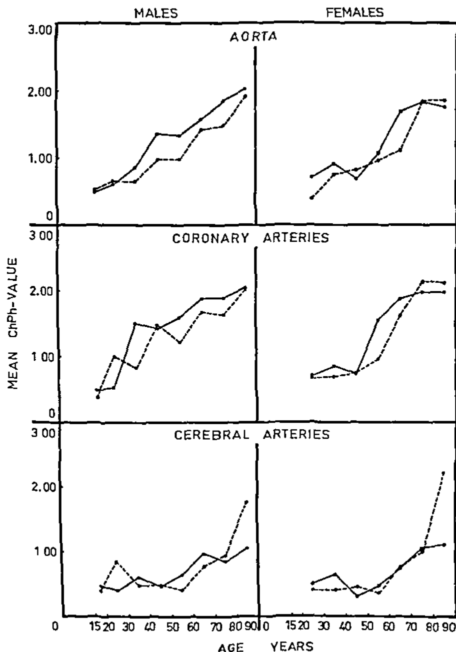


Fig 2

The variation with age of the mean aortic, coronary, and cerebral ChPh-value in males and females without and with malignant disease Individuals with hypertension and atherosclerotic heart disease excluded.

TABLE 2

The Mean ChPh-Value, and the Standard Error of the Mean in Individuals without and with Malignant Disease Total Series

Age-group	Males: Without malignant disease Mean	S E	Males: With malignant disease Mean	S E	Females: Without malignant disease Mean	S E	Females: With malignant disease Mean	S E
				Aorta				
15-19	0.47		0.52					
20-29	0.59		0.66		0.57		0.38	
30-39	1.26	0.21	0.74	0.08	0.98	0.11	0.74	0.04
40-49	1.46	0.11	0.98		1.01	0.10	0.95	0.10
50-59	1.61	0.12	1.21	0.15	1.40	0.09	1.15	0.10
60-69	1.84	0.07	1.53	0.13	1.83	0.06	1.54	0.11
70-79	1.96	0.06	1.82	0.16	2.03	0.05	2.07	0.12
80-89	1.97	0.07	2.01		2.00	0.09	1.91	
				Coronary arteries				
15-19	0.48		0.38					
20-29	0.53		1.00		0.72		0.68	
30-39	2.14	0.24	0.84	0.11	1.12	0.21	0.70	0.14
40-49	1.90	0.19	1.50		1.12	0.24	0.93	0.12
50-59	1.84	0.08	1.34	0.10	1.79	0.15	1.39	0.22
60-69	2.00	0.05	1.91	0.10	2.01	0.07	1.74	0.13
70-79	2.02	0.07	1.90	0.15	2.10	0.08	2.07	0.12
80-89	2.02	0.08	2.09		2.18	0.09	2.27	
				Cerebral arteries				
15-19	0.44		0.40					
20-29	0.39		0.86		0.47		0.40	
30-39	0.79	0.21	0.44	0.04	0.83	0.14	0.46	0.10
40-49	1.00	0.18	0.47		0.51	0.09	0.53	0.09
50-59	1.09	0.14	0.45	0.05	0.96	0.15	0.45	[illegible]
60-69	1.13	0.10	0.88	0.16	1.10	0.13	0.90	0.14
70-79	1.49	0.14	1.26	0.17	1.54	0.13	1.08	0.17
80-89	1.44	0.12	1.55		1.71	0.17	1.50	

As mentioned, *Juhl* (24) found that individuals with malignant disease had less atherosclerosis than other individuals. His series was very great 3,725 cases of malignant disease which were compared to a control group of 8,630 cases, who had died from "all other causes" In a subsequent paper, the applicability of post-mortem material in an epidemiological investigation was studied (*Juhl* (25)) All individuals with atherosclerotic disease, and males with bronchial carcinoma, were excluded from the series It was thereby reduced to 2,961 cancer cases and 3,815 control cases These latter were classified into three groups, namely a "surgical group" comprised of individuals who had died following an operation, a "non-surgical group" where no operation had

TABLE 3

The Mean ChPh Value, and the Standard Error of the Mean, in Individuals without and with Malignant Disease Individuals with Hypertension and Atherosclerotic Heart Disease Excluded

Age group	*Males*				*Females*			
	Without malignant disease		With malignant disease		Without malignant disease		With malignant disease	
	Mean	S E	Mean	S E	Mean	S E	Mean	S E
				Aorta				
15–19	0.47		0.52					
20–29	0.59		0.66		0.71		0.38	
30–39	0.86		0.65		0.92	0.12	0.74	0.04
40–49	1.36		0.98		0.68		0.82	
50–59	1.33	0.15	0.99	0.06	1.07		0.97	
60–69	1.57	0.09	1.43	0.14	1.70	0.08	1.12	
70–79	1.84	0.14	1.48	0.24	1.83	0.12	1.84	0.22
80–89	2.01	0.13	1.91		1.76		1.86	
				Coronary arteries				
15–19	0.48		0.38					
20–29	0.53		1.00		0.71		0.68	
30–39	1.50		0.82		0.86	0.13	0.70	0.14
40–49	1.43		1.50		0.74		0.76	
50–59	1.60	0.13	1.22	0.08	1.56		0.98	
60–69	1.88	0.10	1.68	0.20	1.89	0.12	1.63	
70–79	1.89	0.07	1.64	0.16	1.99	0.22	2.14	0.19
80–89	2.06	0.19	2.04		1.99		2.12	
				Cerebral arteries				
15–19	0.44		0.40					
20–29	0.39		0.86		0.50		0.40	
30–39	0.60		0.47		0.65	0.11	0.39	0.08
40–49	0.46		0.47		0.30		0.44	
50–59	0.65	0.12	0.41	0.04	0.47		0.35	
60–69	0.97	0.15	0.77	0.17	0.74	0.08	0.75	
70–79	0.85	0.23	0.96	0.14	1.06	0.24	1.00	0.28
80–89	1.05	0.11	1.78		1.10		2.23	

been performed and an "accident and suicide group" With the exception of a few age-groups in the latter group, all the control groups showed more atherosclerosis than the cancer groups, and Juhl felt justified to maintain that a negative correlation does exist between cancer and atherosclerosis

Parrish (30) also made a very detailed statistical analysis of his series, 764 cancer cases, and 1,967 non-cancer cases When he compared the cancer group to an "all other causes" group, the former showed less atherosclerosis than the latter When the cancer group was compared to an accident group, however, no difference could be found The

accident group was regarded as the most suitable group for comparison from a statistical point of view. Consequently, Parrish concluded that the difference which appeared when the cancer group was compared to the "all other causes" group constituted an example of Berkson's fallacy, i.e. a result which appears as a consequence of the selection of the series, and that there is no real correlation between cancer and atherosclerosis.

These two studies have been referred to in some detail because both were based on large, retrospective autopsy series, and in both the applicability of such a material in an epidemiological study was discussed. In both, a thorough statistical analysis was made, but the conclusions were quite opposite to each other. Obviously, only one can be correct.

Grosse (20) collected from the literature 15 studies, in all of which it was concluded that individuals with cancer had less atherosclerosis than the control cases without cancer. Grosse did not accept this difference as real, but contended that it is a natural result of the different death rate of individuals with atherosclerotic disease and with malignant disease. He therefore maintained that there only appears to be a correlation between cancer and atherosclerosis. Grosse's views were critisized by *Freudenberg* (17), but *Grosse* (21) maintained his opinion. Freudenberg suggested that the difference in the severity of atherosclerosis between cancer and non-cancer individuals was due to the existence of two constitutional types of individuals, one who develops and dies from cancer, and one who develops severe atherosclerosis and dies from its complications. If this is true, it must of course be nonsense to speak about a correlation between the conditions, in the sense that one "protects" the individual from the other. The reasoning of both Grosse and Freudenberg is difficult to comprehend for one not specially trained in bio statistics. At any rate, it seems that it is still an open question whether or not there is a real correlation between atherosclerosis and cancer.

SUMMARY

The relation of malignant disease to atherosclerosis in the aorta, the coronary, and the cerebral arteries has been examined in 408 individuals, 211 males and 197 females, ranging in age from 15 to 89 years. The total cholesterol/phospholipoid ratio in the arterial wall—the ChPh value—has been used as index for the severity of atherosclerosis.

In the total series, individuals with malignant disease showed less advanced atherosclerosis than individuals without malignant disease. However the latter group was most heavily weighted with individuals with hypertension and atherosclerotic heart disease. When the series was cleared of all individuals with these two conditions the difference between the malignant disease and the control group was no longer quite as obvious, and in the cerebral arteries the difference disappeared

completely There were great individual variations and definite conclusions can hardly be drawn from this study

It is pointed out that the opinions on the relationship between atherosclerosis and cancer are controversial

REFERENCES

1 *Anitschkow N* Pathologische Anatomie und allgemeine Pathologie der Arteriosklerose Comptes Rendus de la Deuxieme Conference Internationale de Pathologie Geographique Utrecht 1934 pp 44–101

2 *Baker A B & Iannone A* Cerebrovascular disease VII A study of etiologic mechanisms Neurology *11* (Part 2) 23 31 1961

3 *Baker A B Kinnard J & Iannone A* Cerebrovascular disease VIII Role of nutritional factors Neurology *11* 380–389 1961

4 *Björnsson J* Arteriosclerosis Munksgaard Copenhagen 194?

5 *Burkhardt L* Uber Beziehungen zwischen Bronchuscarcinom und Atherosklerose Med Klin *54* 1613–1614 1959

6 *Busch M* Das Verhalten der Aorta bei Krebsträgern Zentralbl f allg Path u path Anat *34* 614 19?4

7 *Bahr E* Die Atherosklerose der Herzkranzgefasse in ihrer Beziehung zu Alter Krankheit und Konstitution Arch f Kreislaufforsch *3* 95–124 1938

8 *Casper J* Bosartige Geschwulste und Arteriosklerose Ztschr f Krebsforsch *36* 354 359 1932

9 *Creed D L Baird W F & Fischer E R* *The severity of aortic atherosclerosis* in certain diseases A necropsy study Am J M Sc *230* 385–391 1955

10 *Dormanns E & Emminger E* Vergleichende Untersuchungen uber Ausbreitung und Starke der Atherosklerose an 1000 Leichen von uber 20 Jahren alten Personen mit besonderer Berucksichtigung von Krebs Tuberkulose und Lues Virchows Arch f path Anat *?93* 545 550 1934

11 *Dungal N & Benediktsson T* Gastric cancer and atherosclerosis Lancet *1* 931 932 1958

12 *Elkeles A* Radiological studies of the abdominal aorta in individuals over 50 as an aid to the differential diagnosis between benign and malignant ulceration of the stomach Brit J Radiol *22* 280–283 1949

13 *Elkeles A* Peptic ulcer in the aged and gastric carcinoma in their relationship to atherasclerosis Am J Roentgenol *70* 797 803 1953

14 *Elkeles A* Cancer and atherosclerosis Brit J Cancer *10* ?47 250 1956

15 *Elkeles A* A comparative radiological study of calcified atheroma in males and females over 50 years of age Lancet *2* 714 715 1957

16 *Elkeles A* Calcified atherosclerosis and cancer Brit J Cancer *13* 403 40? 1959

17 *Freudenberg K* Arteriosklerose und Krebs Zeitschr f Krebsforsch *62* 527 536 1958

18 *Giertsen J Chr* Atherosclerosis in an autopsy series 1 Chemical grading of atherosclerosis Acta path et microbiol scandinav Supplementum *170* 1964

19 *Giertsen J Chr* Atherosclerosis in an autopsy series 3 Inter relationship between atherosclerosis in the aorta the coronary and the cerebral arteries Acta path et microbiol scandinav *63* 391 413 1965

20 *Grosse H* Arteriosklerose und Krebs Ztschr f Krebsforsch *6?* 519 526 1958

21 *Grosse H* Arteriosklerose und Krebs Ztschr f Krebsforsch *6?* 537 539 1958

22 *Hyman J B & Epstein F H* A study of the correlation between roentgenographic and post mortem calcification of the aorta Am Heart J *48* 540 543 1954

23 *Janssen W Hevelke G Mielke H G & Schut F H* Leukamie und Arteriosklerose Ztschr f d ges inn Med u d Grenzgeb *12* 543 547 1957

24 *Juhl S* Cancer and atherosclerosis Negative correlation Acta path et micr biol scandinav *37* 166 18 1955

25 *Juhl S* Cancer and atherosclerosis ? Applicability of postmortem statistics in the study of the negative correlation Acta path et m crob ol scand nav *41* 99 104 1957

26 *Lober, P H* Pathogenesis of coronary sclerosis Arch Path 55 357-383 1953
27 Manual of the International Statistical Classification of Diseases Injuries and Causes of Death WHO Genf 1949
28 *Meriggi, A* Sui rapporti fra aterosclerosi aortica e cancro polmonare Riv Anat Pat Oncol *17* 677-694 1960 Cit English Summary
29 *Moran E Schwartz A & Ungar, H* A correlative study between the radiologic and pathologic diagnosis of atherosclerosis of the aorta Am Heart J *65* 190-194 1963
30 *Parrish H M* Epidemiology of ischemic heart disease among white males I Relationship between coronary atherosclerosis and cancer of various sites J Chronic Dis *14* 311-325 1961
31 *Schneider, E* Arteriosklerose und maligne Tumoren Med Klin 32 1565 1566 1936
32 *Sjovall H & Wihman G* Beobachtungen uber die Arteriosklerose in Schweden samt einem Beitrag zur Frage der Lipoidose der Arterienintima Acta path et microbiol scandinav Supplementum *20* 1934
33 *Spain D M, Greenblatt I J Snapper I & Cohn T* The degree of coronary and aortic atherosclerosis in necropsied cases of multiple myeloma Am J M Sc *231* 165-167, 1956
34 *Tichy, J* Rakovina ve vztahu k chorobám s porušenou výměnou cholesterolu—

Acta path et microbiol scandinav 66 351 358 1966

The University of Bergen School of Medicine the Gade Institute
Department of Pathology, Bergen, Norway (Head Erik Waaler, M D)

ATHEROSCLEROSIS IN AN AUTOPSY SERIES

9 *Relation of Atherosclerotic Heart Disease to Atherosclerosis*

By

J Chr Giertsen

Received 8 x 65

A sharp distinction should of course be made between atherosclerosis and atherosclerotic disease, *i e*, the clinical manifestations of atherosclerosis Clinical studies of atherosclerosis are hampered by the fact that the severity of atherosclerosis cannot be assessed in the living individual A clinical study must therefore deal with patients who suffer from atherosclerotic disease, and compare these to healthy control individuals These are, however, only healthy insofar as they have no atherosclerotic disease, they certainly have atherosclerosis As *Holman, McGill Jr, Strong & Geer* (7) pointed out, we must probably accept atherosclerosis in adults as "normal" in a statistical sense Do "normal" control individuals really have less atherosclerosis than those who develop atherosclerotic disease, so that inferences with regard to the severity of atherosclerosis, per se, can be made from a comparison like the aforementioned?

Ackerman, Dry & Edwards (1) found that the clinical diagnosis of coronary insufficiency paralleled to a remarkable degree the number of hearts with a severe coronary atherosclerosis Other authors have also found that a severe degree of atherosclerosis is necessary to produce atherosclerotic heart disease (*Lober* (8)), or to form the basis for a thrombus (*Spain & Bradess* (10)) On the other hand, *Baker, Iannone & Kinnard* (2) found that a patient may have a minimal degree of coronary atherosclerosis, and nevertheless a fatal thrombus may be formed *Parrish* (9) and *Strong & McGill Jr* (11) made similar observations

MATERIAL

[illegible]

of atherosclerosis (*Giertsen* (3))

The series was divided into two main groups viz individuals without and with atherosclerotic heart disease i e angina pectoris and/or myocardial infarction recent or old Individuals with chronic passive congestion of possible atherosclerotic aetiology, but without definite ischaemic symptoms have not been included in the

atherosclerotic heart disease group The term atherosclerotic heart disease has been used because the frequently used terms coronary heart disease and ischaemic heart disease are considered to be broader terms, which do not necessarily indicate an atherosclerotic aetiology

Thereafter, the series was cleared of all individuals with hypertension and malignant disease, and the remaining series studied

Both main groups were sub-divided into age and sex groups For each group the mean ChPh value was calculated and for groups of 6 or more cases also the standard error of the mean

RESULTS

Table 1 shows the age and sex distribution of individuals without and with atherosclerotic heart disease It appears that about half the number of males, and slightly more than one third of the females in the total series had suffered from atherosclerotic heart disease Only 6 of these had had angina pectoris alone No male below 40, and no female below 50 years of age had atherosclerotic heart disease The table also shows that the series is considerably reduced when it is cleared of individuals with hypertension and malignant disease, and many groups in the cleared series are poorly represented The reduction is, proportionately, most marked in the number of females

TABLE I

The Age and Sex Distribution of Individuals above 40 Years of Age without and with Atherosclerotic Heart Disease (AHD)

Age group	Males		Females	
	Without AHD	With AHD	Without AHD	With AHD
	Total series			
40–49	14	5		
50–59	29	8	28	3
60–69	32	25	40	8
70–79	34	15	34	19
80–89	17	8	12	12
Total	126	61	114	42
	Individuals with hypertension and malignant disease excluded			
40–49	3	3		
50–59	9	3		
60 69	8	9	11	3
70–79	8	5	7	4
80–89	7	4	3	3
Total	35	24	21	10

Table 2 and Fig 1 show that individuals of both sexes with atherosclerotic heart disease have a considerably higher ChPh value than individuals without atherosclerotic heart disease in the younger age groups This is especially true in females The difference levels off

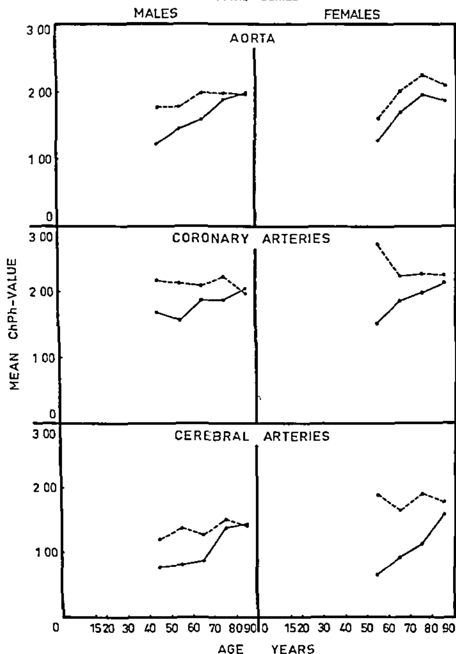


Fig 1

The variation with age of the mean aortic coronary and cerebral ChPh value in males and females without and with atherosclerotic heart disease Total series

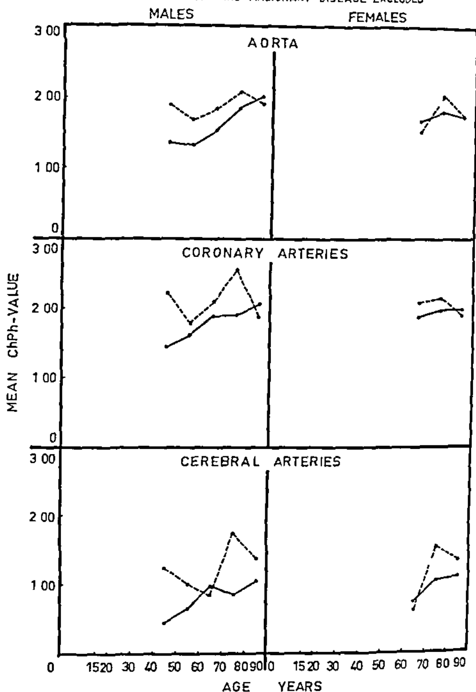


Fig 2

The variation with age of the mean aortic coronary and cerebral ChPh value in males and females without and with atherosclerotic heart disease Individuals with hypertension and malignant disease excluded

with age, and in the ninth decade, males with atherosclerotic heart disease have a lower ChPh value than males without, even in the coronary arteries

As shown in Table 3 and Fig 2, approximately the same difference between the two main groups is present in the cleared series The curves are more irregular, however, and the difference is most marked in males Moreover, in the ninth decade females with atherosclerotic heart disease also have a lower coronary ChPh value than females without this disease

TABLE 2

The Mean ChPh Value and the Standard Error of the Mean in Individuals above 40 Years of Age without and with Atherosclerotic Heart Disease (AHD) Total Series

Age group	*Males*				*Females*			
	Without AHD		With AHD		Without AHD		With AHD	
	Mean	S E	Mean	S E	Mean	S E	Mean	S E
				Aorta				
40–49	1 21	0 09	1 77					
50–59	1 44	0 10	1 78	0 18	1 27	0 07	1 62	
60–69	1 58	0 06	1 99	0 10	1 69	0 07	2 01	0 14
70–79	1 89	0 08	1 99	0 10	1 96	0 07	2 19	0 07
80–89	1 99	0 10	1 95	0 11	1 87	0 10	2 11	0 13
				Coronary arteries				
40–49	1 69	0 16	2 17					
50–59	1 57	0 09	2 14	0 15	1 53	0 12	2 72	
60–69	1 86	0 06	2 11	0 07	1 86	0 07	2 26	0 14
70–79	1 87	0 07	2 24	0 13	1 99	0 07	2 27	0 11
80–89	2 05	0 10	1 97	0 10	2 13	0 12	2 26	0 12
				Cerebral arteries				
40–49	0 78	0 16	1 21					
50–59	0 81	0 11	1.39	0 35	0 66	0 08	1 89	
60–69	0 88	0 10	1 29	0 14	0 92	0 10	1 65	0 28
70–79	1 38	0 14	1 52	0 16	1 13	0 12	1 93	0 16
80–89	1 46	0 12	1 44	0 22	1 59	0 24	1 77	0 21

The individual variation within the groups was great and there were several individuals with atherosclerotic heart disease who had a lower ChPh value than individuals without athersclerotic heart disease, *i e*, there was a considerable overlapping between the two main groups

Thrombosis was found in the coronary arteries on gross examination in 32 males and 16 females with atherosclerotic heart disease, that is in 52 and 38 per cent, respectively Twenty-four of these males were younger than 60 years of age 16 had a ChPh value lower than,

and 16 a value higher than 2.00 In contrast to this, 14 of the females were older than 60 years of age, and 13 had a ChPh value higher than 2.00

TABLE 3

The Mean ChPh Value and the Standard Error of the Mean in Individuals above 40 Years of Age without and with Atherosclerotic Heart Disease (AHD) Individuals with Hypertension and Malignant Disease Excluded

Age group	*Males*				*Females*			
	Without AHD		With AHD		Without AHD		With AHD	
	Mean	S E	Mean	S E	Mean	S E	Mean	S E
				Aorta				
40–49	1.36		1.90					
50–59	1.33	0.15	1.69					
60–69	1.57	0.09	1.83	0.09	1.70	0.08	1.54	
70–79	1.84	0.14	2.07		1.83	0.12	2.04	
80–89	2.01	0.13	1.92		1.76		1.77	
				Cerebral arteries				
40–49	1.43		2.22					
50–59	1.60	0.13	1.79					
60–69	1.88	0.10	2.08	0.16	1.89	0.12	2.08	
70–79	1.89	0.07	2.54		1.99	0.22	2.14	
80–89	2.06	0.19	1.88		1.99		1.91	
				Coronary arteries				
40–49	0.46		1.25					
50–59	0.65	0.12	1.01					
60–69	0.97	0.15	0.84	0.11	0.74	0.08	0.63	
70–79	0.85	0.23	1.74		1.06	0.24	1.54	
80–89	1.05	0.11	1.39		1.10		1.34	

DISCUSSION

The basic lesion necessary to produce atherosclerotic heart disease is of course atherosclerosis, and it seems natural that the functional impairment of the coronary circulation should depend more on the degree of narrowing of the lumen which is produced than on the extent of the process in the arteries Consequently, when the grading method of atherosclerosis is based on the degree of narrowing as in the studies of *Ackerman, Dry & Edwards* (1) and *Lober* (8) a very close correlation between the degree of atherosclerosis and atherosclerotic heart disease must be expected These authors did not distinguish between narrowing of the lumen produced by the atherosclerotic plaque itself or by a complicating factor, but *Baker Iannone & Kinnard* (2) found that even a minimal degree of atherosclerosis might form the basis for a complicating and fatal thrombosis

The findings in the present study seem to be more in agreement with this Obviously, a severe degree of atherosclerosis, i e, severe in extent, is not a prerequisite to precipitate a functional disturbance A complication seems to be necessary in many instances The distribution of gross thrombosis showed a peculiar sex difference in this series, but too much attention should not be paid to this difference The real frequency of thrombosis cannot be evaluated, because systematic microscopical examination of the arteries has not been carried out

The 3 normotensive 40-year-old males with atherosclerotic heart disease had a very high mean coronary ChPh-value The actual values were 1 31, 2 86, and 2 50 The corresponding heart weights were 575, 420, and 500 g, all of which are suggestive of cardiac hypertrophy All three had suffered a previous infarction, and died from a new infarction These males may in fact have been hypertensive, and thus examples of erroneous blood pressure classification, as previously discussed (*Giertsen* (5)) At any rate, they clearly demonstrate how significant such doubtful cases may be in a series in which the groups are small

Very severe atherosclerosis was found in many old individuals who had not suffered from any functional impairment of the coronary circulation The gradual loss of elasticity in the coronary arteries with subsequent dilatation, which occurs in old age, and the development of anastomoses, which especially occurs in severe atherosclerosis (*Gould* (6)), may help to prevent such an impairment

It may be concluded that individuals with atherosclerotic heart disease will, on an average, have more atherosclerosis than individuals without this disease, except in old age There are great individual variations, however, and an individual without any impairment of the coronary circulation may have just as much, or even more atherosclerosis than one who has a myocardial infarction

SUMMARY

The relation of atherosclerotic heart disease to atherosclerosis in the aorta, the coronary, and the cerebral arteries has been examined in 408 individuals, 211 males and 197 females, ranging in age from 15 to 89 years The total cholesterol phospholipoid ratio in the arterial wall—the ChPh-value— has been used as index for the severity of atherosclerosis

Atherosclerotic heart disease (angina pectoris and/or myocardial infarction) was found in males 40 to 89 years of age, and in females 50 to 89 years of age Consequently, the study was limited to these age-groups

In the total series individuals with atherosclerotic heart disease showed, on an average, more atherosclerosis than individuals without

this disease, especially in the younger age groups and in females The difference leveled off with age, and disappeared in the ninth decade Essentially the same was found when the series was cleared of individuals with hypertension and malignant disease, except that the difference between the two groups was most pronounced in males

There was considerable overlapping of the ChPh values in the two groups Many individuals with atherosclerotic heart disease displayed a comparatively mild degree of atherosclerosis whereas many individuals without any functional impairment of the coronary circulation showed a very severe degree of atherosclerosis A complication of atherosclerosis seems necessary in many instances to precipitate functional impairment Gross thrombosis was found in 52 per cent of the males and in 38 per cent of the females with atherosclerotic heart disease most frequently below 60 years of age in males and above this age in females *The real frequency of thrombosis could not, however, be evaluated in this series*

REFERENCES

1 *Ackerman R F Dry T J & Edwards J E* Relationship of various factors to the degree of coronary atherosclerosis in women Circulation *1* 1345 1354 1950

2 *Baker A B Iannone A & Kinnard J* Cerebrovascular disease VI Relationship to disease of the heart and the aorta Neurology *11* 63 70 1961

3 *Giertsen J Chr* Atherosclerosis in an autopsy series 1 Chemical grading of atherosclerosis Acta path et microbiol scandinav Supplementum *170* 1964

4 *Giertsen J Chr* Atherosclerosis in an autopsy series 3 Inter relationship between atherosclerosis in the aorta the coronary and the cerebral arteries Acta path et microbiol scandinav *63* 391 413 1965

5 *Giertsen J Chr* Atherosclerosis in an autopsy series 7 Relation of hyper tension to atherosclerosis Acta path et microbiol scandinav *66* 331 340 1966

6 *Gould S E* Diseases of the coronary arteries In Gould S F (Ed) Pathology of the heart Second Ed 1960 Charles C Thomas Springfield Ill pp 558 and 580

7 *Holman R L McGill Jr H C Strong J P & Geer J C* The natural history of atherosclerosis The early aortic lesions as seen in New Orleans in the middle of the 20th century Am J Path *34* 209 229 1958

8 *Lober P H* Pathogenesis of coronary sclerosis Arch Path *55* 357 383 1953

9 *Parrish H M* Epidemiology of ischemic heart disease among white males III Role of coronary atherosclerosis and clot formation in patients with ischemic heart disease J Chronic Dis *14* 339 354 1961

10 *Spain D M & Bradess V A* The relationship of coronary thrombosis to coronary atheroschlerosis and ischemic heart disease Am J M S *240* 701 710 1960

11 *Strong J P & McGill Jr H C* The natural history of coronary atherosclerosis Am J Path *40* 37–49 1962

Acta path. et microbiol. scandinav. 66: 359-368, 1966

The Section of Virology, Department of Clinical Bacteriology,
Karolinska sjukhuset, Stockholm 60

THERMOLABILITY AND MERCAPTOETHANOL SENSITIVITY OF ADENOVIRUS NEUTRALIZING ACTIVITY IN IMMUNE AND NORMAL ANIMAL SERA

By

GUNVOR SVARTZ-MALMBERG

Received 21.viii.65

During a study of adenovirus antibodies in children (*Sterner & Svartz-Malmberg* 1962) it was observed that some sera possessed a higher neutralizing activity before than after heating at 56° C for 30 minutes (heat inactivation). Investigations carried out with sera from children and adults confirmed this finding. The thermolabile neutralizing activity was most frequently demonstrated in the neutralization of adenovirus type 7 (*Svartz-Malmberg* 1963 a).

Experiments were then started with rabbits and guinea pigs injected with adenovirus type 7 antigen. In sera collected during the first period (1–2 weeks) after inoculation a rise of thermolabile neutralizing activity was found to precede the appearance of thermostable antibodies (*Svartz-Malmberg* 1964).

The early appearance of antibodies sensitive to treatment with mercaptoethanol has been demonstrated by *Bauer, Mathies & Stavitsky* (1963) after immunization with proteins, Salmonella antigens and phage, and by *Berlin* (1963) and *Svehag* (1964 a) after immunization with animal viruses. In the present report the influence of mercaptoethanol is compared with the above mentioned effect of heat treatment on the neutralizing activity to adenovirus type 7 in early immune sera.

MATERIALS AND METHODS

[illegible]

serum and 60 per cent of Parker's Medium 199 containing penicillin and streptomycin. After washing three times in phosphate buffered saline the cultures were

---

My thanks are due to Mr S. *Hedman* of the King Gustaf V Research Institute, Stockholm, for performing the ultracentrifugal analyses. The skilful technical assistance of Mrs *Marianne* [illegible], Mrs [illegible] and Mr [illegible] is gratefully acknowledged.

maintained in Eagle's Minimum Essential Medium without any addition of serum They were *inoculated* with a large dose of virus and when the degeneration was complete the tissue culture fluid was harvested and stored at —25° C Following centrifugation for 45 minutes at 3000 r p m it was used for immunization as well as for the neutralization tests One rabbit received antigen pretreated with fluorocarbon (Freon 113) in cold The titres of the antigens were in the range of 10^{4}–$10^{?}$ TCD_{50}/ml as recorded on the 14th day after inoculation of the cell cultures

Immunization procedure Adult rabbits were immunized by a single intravenous injection of 10 ml *antigen each During the following 4 14 days blood* specimens were collected almost daily by puncture of the ear vein. Exsanguination was done from the jugular artery The blood was allowed to clot for a maximum of 3 hours and was then centrifuged at +4° C Sera were dispensed in small quantities and stored in Resco deep freeze units at either —60° C or —74° C. *Once a portion had* been thawed it was never reused except for making a heat inactivated sample Adult guinea pigs were injected *intravenously with 2 ml of antigen each In most* experiments different animals were bled to death at different intervals after inoculation *owing to the difficulty of getting enough test material when bleeding the same* animal almost daily Guinea pigs were always bled by heart puncture The sera were separated and stored *in* the same manner as the rabbit sera

Assay of virus neutralizing activity The method of *Kjellen et al* (1957) was employed HeLa cells maintained in Eagle's Minimum Essential Medium without any addition of serum were incubated in a roller drum at 35° C The medium was changed after one week and the cell cultures could usually be kept in good condition for 14 days after inoculation All serum samples except the mercaptoethanol treated ones and their controls were thawed in an ice bath immediately before mixing them with the virus antigen All mixtures were kept in an ice bath until the incubation which was carried out in a water bath at 37° C for 60 minutes The virus antigen was used in a dilution which produced a cytopathic effect on the first or second day after inoculation Most antigens were diluted 1 10 The serum virus mixtures were inoculated into duplicate tubes The day on which a cytopathic effect was detectable in the virus control was taken as day 0 For each serum sample the number of days including that on which the first signs of *specific* degeneration were observed was recorded as a measure of neutralizing activity Usually the cytopathic changes *appeared simultaneously in the two tubes inoculated* with *identical serum virus mixtures in cases in which the difference exceeded two days* a mean value was *approximated If a cytopathic effect* was *detected in the virus control* on the first day after inoculation and if a serum protected the cells throughout 14 days its neutralizing activity was recorded as >13

Heat inactivation Serum samples heated at 56° C for 30 minutes are referred to as heat inactivated samples

Mercaptoethanol treatment The serum which was diluted 1 2 in phosphate buffered saline (pH 7 4) was mixed with 2 mercaptoethanol *diluted to 1 M in the* same buffer to a final concentration of 0 1 M After two hour s incubation at room temperature the samples were dialyzed at +4° C against phosphate *buffered saline* As control a sample of the serum was kept at room temperature for two hours without addition of mercaptoethanol and then dialyzed for the same period *The addition* of iodoacetamide commonly used for the prevention of reaggregation of mercaptoethanol dissociated proteins to the dialyzing medium had to be omitted because of the strong toxic effect exerted on the cells by preparations dialyzed against this substance The samples were tested both in the original dilution of 1 2 and in most cases in dilution 1 4 as well

Assays of complement activity Employing the *Fulton Dumbell* technique (1949) as modified by *Svedmyr et al* (1953) the complement activity of native guinea pig serum was assayed in the same way as complement is titrated for control of the anticomplementary action of antigens One drop of serial twofold dilutions of complement in barbiturate buffer starting from 1 5 was added to two drops of barbiturate buffer one of them being substituted for the antigen The mixture was kept overnight at +4° C On the following day two drops of haemolytic system were added before the final incubation at 37° C for two hours

Analytic ultracentrifugation Analyses were carried out by Mr S *Helman* of the King Gustaf V *Research Institute* Stockholm The ultracentrifuge of type Beckman Spinco E was run for two hours at 59 000 to 60 000 r p m

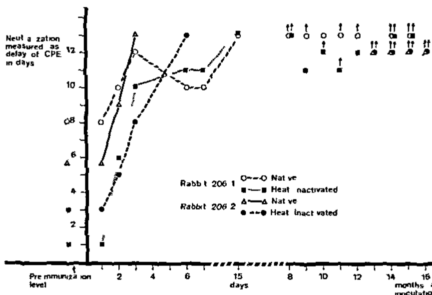


Fig 1

The development of neutralizing activity in rabbits immunized with a single i tion of adenovirus type 7 antigen Sera were tested in dilution 1 2 Arrows a the symbol sign fy that the value may be higher than indicated

RESULTS

1 *Investigations of Sera from Immunized Animals*

Early immune sera from rabbits and guinea pigs were previo found to possess a higher neutralizing activity before than after tr ment at 56° C for 30 minutes (*Svartz Malmberg* 1964) This is illustrated by the data in Fig 1 The human sera in which a sim heat sensitivity of adenovirus type 7 antibodies was observed howe were not obtained during or immediately after infection (*Svart- Ma berg* 1963 a) In order to study the properties of antibodies foun long time after immunization sera were collected from the two rab in Fig 1 over a period of 15 and 16 months respectively after a si injection of antigen Once maximum levels of neutralizing activity w reached the serum specimens were capable of protecting the cells fr cytopathic effects throughout the period of the neutralization test significant influence of heat inactivation being demonstrable The n tralizing activity was surprisingly high and actually the pool of serum volumes not needed for the investigations will supply the lab atory for a long time with adenovirus type 7 typing serum since it be used for this purpose in a dilution of 1 80

Consequently, heat sensitive neutralizing activity was demonstra in rabbits only during the first days after immunization The same t of early response was found also in guinea pigs Investigations w

maintained in Eagle's Minimum Essential Medium without any addition of serum They were inoculated with a large dose of virus and when the degeneration was complete the tissue culture fluid was harvested and stored at —25° C. Following centrifugation for 45 minutes at 3000 r p m it was used for immunization as well as for the neutralization tests One rabbit received antigen pretreated with fluorocarbon (Freon 113) in cold The titres of the antigens were in the range of 10^{6}–10^{7} TCD_{50}/ml as recorded on the 14th day after inoculation of the cell cultures

Immunization procedure Adult rabbits were immunized by a single intravenous injection of 10 ml antigen each During the following 4 14 days blood specimens were collected almost daily by puncture of the ear vein Exsanguination was done from the jugular artery The blood was allowed to clot for a maximum of 3 hours and was then centrifuged at +4° C Sera were dispensed in small quantities and stored in Revco deep freeze units at either —60° C or —74° C Once a portion had been thawed it was never reused except for making a heat inactivated sample Adult guinea pigs were injected intravenously with 2 ml of antigen each In most experiments different animals were bled to death at different intervals after inoculation owing to the difficulty of getting enough test material when bleeding the same animal almost daily Guinea pigs were always bled by heart puncture The sera were separated and stored in the same manner as the rabbit sera

Assay of virus neutralizing activity The method of *Kjellén et al* (1957) was employed HeLa cells maintained in Eagle s Minimum Essential Medium without any addition of serum were incubated in a roller drum at 35° C. The medium was changed after one week and the cell cultures could usually be kept in good condition for 14 days after inoculation All serum samples except the mercaptoethanol treated ones and their controls were thawed in an ice bath immediately before mixing them with the virus antigen All mixtures were kept in an ice bath until the incubation which was carried out in a water bath at 37° C for 60 minutes The virus antigen was used in a dilution which produced a cytopathic effect on the first or second day after inoculation Most antigens were diluted 1 10 The serum virus mixtures were inoculated into duplicate tubes The day on which a cytopathic effect was detectable in the virus control was taken as day 0 For each serum sample the number of days including that on which the first signs of specific degeneration were observed was recorded as a measure of neutralizing activity Usually the cytopathic changes appeared simultaneously in the two tubes inoculated with identical serum virus mixtures In cases in which the difference exceeded two days a mean value was approximated If a cytopathic effect was detected in the virus control on the first day after inoculation and if a serum protected the cells throughout 14 days its neutralizing activity was recorded as >13

Heat inactivation Serum samples heated at 56° C for 30 minutes are referred to as heat inactivated samples

Mercaptoethanol treatment The serum which was diluted 1 2 in phosphate buffered saline (pH 7 4) was mixed with 2 mercaptoethanol diluted to 1 M in the same buffer to a final concentration of 0 1 M After two hour s incubation at room temperature the samples were dialyzed at +4° C against phosphate buffered saline As control a sample of the serum was kept at room temperature for two hours without addition of mercaptoethanol and then dialyzed for the same period The addition of iodacetamide commonly used for the prevention of reaggregation of mercaptoethanol dissociated proteins to the dialyzing medium had to be omitted because of the strong toxic effect exerted on the cells by preparations dialyzed against this substance The samples were tested both in the original dilution of 1 2 and in most cases in dilution 1 4 as well

Assays of complement activity Employing the Fulton Dumbell technique (1949) as modified by *Svedmyr et al* (1953) the complement activity of native guinea pig serum was assayed in the same way as complement is titrated for control of the anticomplementary action of antigens One drop of serial two fold dilutions of complement in barbiturate buffer starting from 1 5 was added to two drops of barbiturate buffer one of them being substituted for the antigen The mixture was kept overnight at +4° C On the following day two drops of haemolytic system were added before the final incubation at 37° C for two hours

Analytic ultracentrifugation Analyses were carried out by Mr S *Hedman* of the King Gustaf V Research Institute Stockholm The ultracentrifuge of type Beckman Spinco E was run for two hours at 59 000 to 60 000 r p m

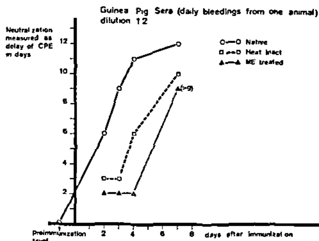


Fig 3

Comparison of the influence of heat inactivation and mercaptoethanol treatment on early immune serum specimens collected from one guinea pig

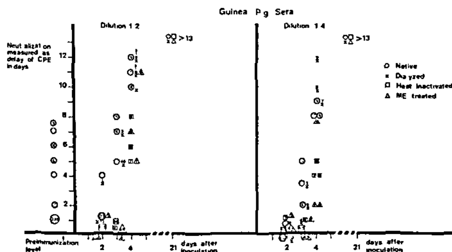


Fig 4

Comparison of the influence of heat inactivation and mercaptoethanol treatment on early immune sera from guinea pigs Each figure within or above a symbol indicates an individual animal Arrows above the symbol signify that the value may be higher than indicated

in buffer restored the neutralizing capacity lost during heat inactivation No apparent effect was however, obtained by such addition of complement to mercaptoethanol-treated nor to heated samples of rabbit or guinea pig sera

Complement activity was found to be sensitive to mercaptoethanol treatment When tested with sensitized sheep erythrocytes by the

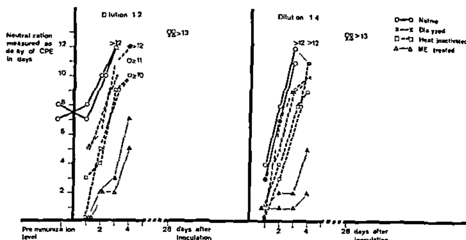


Fig 2

Comparison of the influence of heat inactivation and mercaptoethanol treatment on early immune sera from rabbits
The late serum (28 days) collected from different animals

undertaken to find out whether mercaptoethanol treatment would give rise to effects similar to those arrived at by heat inactivation In the previous experiments any unnecessary loss of time had carefully been avoided after a serum sample had once been taken from the deep freeze unit However, as the mercaptoethanol procedure involved treatment of the thawed specimen for about 20 hours, control samples treated identically, except for the addition of mercaptoethanol, were included and are referred to as 'dialyzed samples' In contrast to late sera, early immune sera were found to be sensitive to mercaptoethanol treatment as well as to heat inactivation (Figs 2, 3 and 4) In the rabbits values for dialyzed samples tend to lie below those for the non-heated native ones, *as do the values for the mercaptoethanol-treated samples* when compared to the heat inactivated (Fig 2) This might simply be due to some effect of the dialysis On the other hand the change of the serum proteins brought about by heat or by mercaptoethanol might of course be quantitatively or qualitatively different

Similar results were obtained from several bleedings of the same guinea pig, as seen in Fig 3 Data from separate animals exsanguinated in order to get sufficient amounts of serum for the different procedures under investigation tend to give rather inconclusive results as individual variations seem to be more pronounced among guinea pigs than among rabbits (Fig 4)

Some first-day sera were inoculated into HeLa cell cultures In no instance was there evidence of circulating infective virus

With some of the human sera investigated earlier *it was demonstrated* that dilution 1 2 in fresh guinea pig serum (complement), instead of

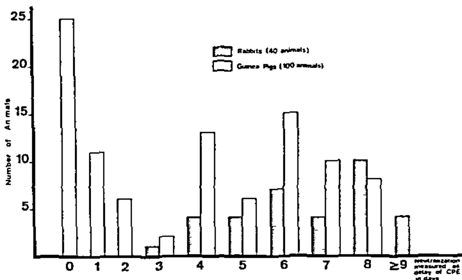


Fig 6

Neutralizing activity to adenovirus type 7 in native samples of 40 normal rabbit and 100 normal guinea pig sera

saline only Native as well as mercaptoethanol treated and dialyzed early immune sera from two rabbits were subjected to analytical ultra centrifugation The samples were divided after treatment and one part was used for centrifugation, the other for a neutralization test Representative ultracentrifugation diagrams are given in Fig 5 The delay of cytopathic effects was reduced from 7 days to one day in the sample still containing 8 4 per cent macroglobulins after treatment and from 10–12 days to 2–3 days in the one with barely visible macroglobulin peak, which has been calculated at 3 5 per cent

2 *Investigations of Normal Sera*

From all animals employed for the experiments a preimmunization sample was collected Normal non heated rabbit sera always possessed a moderate neutralizing activity to adenovirus type 7 Among the guinea pigs about 40 per cent of the sera gave no or very slight neutralization, whereas *some sera were as potent as the rabbit sera* (*Fig 6*) Whether the guinea pigs with negative sera had been differently caged by the breeder or belonged to a certain strain or had some other characteristic in common, has not been possible to elucidate But at all events negative sera and sera having a high neutralizing capacity did not seem to be randomly distributed among the groups of animals bled on different occasions There seems to be no clear-cut correlation between pre- and postimmunization levels in individual animals (*e g* sera nos 4 5 and 6 in Fig 4)

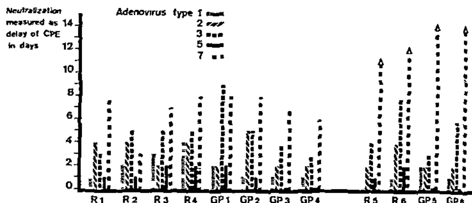


Fig 7

Cross reactivity of native samples of normal and immune sera

Nos R 1–4 normal rabbit sera
Nos GP 1–4 normal guinea pig sera
Nos R 5 R 6 late immune sera from rabbits
Nos *GP 5 GP 6* *late immune sera from guinea pigs*

Arrow shaped columns signify that the value may be higher than indicated

Normal sera were found to react in the same way as early post immunization sera when exposed to either heat inactivation or mercaptoethanol

The cross-reactivity with some other adenovirus types was determined in native normal and immune sera Representative data are given in Fig 7 Not unexpectedly type 3, known to be antigenically related to type 7, is inhibited, in normal sera sometimes as much as type 7 itself Type 5 was practically not influenced at all, whereas occasional reactions were observed with types 1 and 2

DISCUSSION

The neutralization method used in the present investigation offers the advantage of allowing very low serum dilutions (1 2 or 1 4) to be tested against high virus doses Thereby the influence of a heat sensitive factor present in low concentrations can be revealed If the thermolability of normal and early immune sera depends upon a factor separate from the antibodies and efficient only at low dilutions a possible corresponding effect in late sera can obviously not be demonstrated by the method employed, as here the thermostable antibodies will protect the cells throughout the whole test period

The sensitivity to mercaptoethanol does not permit a definite conclusion as to whether the macroglobulin (γM) fraction is really responsible for the early neutralizing activity demonstrated in this investigation By analogy with the results of other authors this might be inferred but direct evidence can be provided only by separation studies which are in progress Human macroglobulins dissociated into lighter

fractions by mercaptoethanol were found to become reaggregated during dialysis unless an alkylating agent was added (*Deutsch & Morton* 1957) The ultracentrifugal analyses of the early adenovirus antisera presented here apparently imply that the influence of mercaptoethanol treatment on neutralization is not correlated to the amount of macroglobulin demonstrable after dialysis The results could be explained by a blocking by mercaptoethanol of the antibody combining site It might also be possible that reaggregated proteins do not regain full neutralizing capacity

Complement dependence of early antibodies against bacteriophages has been reported by *Toussaint & Muschel* (1962) In the present study it was demonstrated that the haemolytic function of complement is destroyed by mercaptoethanol Complement or some other heat labile accessory factor may be necessary for the action of early antibody However, the heat lability and the mercaptoethanol sensitivity of the early neutralizing activity in animal sera cannot be ascribed solely to complement, as it could not be restored by addition of complement This is in agreement with the findings of *Svehag* (1964 b) Apparently the neutralizing principle of normal sera and early immune sera is like complement, sensitive to heat as well as to mercaptoethanol It should be emphasized, though, that the neutralizing capacity of some heated human sera was enhanced to the levels of the corresponding native samples by the addition of complement (*Svartz Malmberg* 1963 a) According to preliminary investigations different human sera also behave differently after heat inactivation and after decomplementation by adsorption to an antigen antibody precipitate (*Svartz-Malmberg* 1963 b) The action of complement on the neutralization reactions will be submitted to further investigations

The cross reactivity with other adenovirus types was found to be similar in normal and immune sera Neutralization of the antigenically related type 3 is found in most sera, sometimes attaining the same levels as type 7 inhibition The slight reactions of some sera with types 1 and 2 cannot be explained by any known antigenic similarity, but might possibly be the results of earlier contact with these or related antigens

The reactions to heat and to mercaptoethanol are similar for normal and early immune sera This makes it difficult to determine an exact point at which the neutralizing activity should properly be called early antibody activity *Svehag* (1964 b) assumed the neutralizing activity of normal sera to be associated with "repeated stimulation with minute amounts of antigen' According to this hypothesis there is apparently no fundamental difference between what is called normal antibody and early immune antibody, which, when compared in the neutralization of adenovirus were also shown to have more properties in common than do early and late immune antibodies

SUMMARY

A rise of thermolabile as well as mercaptoethanol sensitive neutralizing activity was demonstrated in rabbits and guinea pigs during the first days after a primary injection with adenovirus type 7 antigen

In normal rabbit sera and in about 60 per cent of normal guinea pig sera an activity possessing similar properties was detectable

The addition of guinea pig complement did not result in a rise of the neutralizing capacity of animal sera treated with heat or with mercaptoethanol, in contrast to earlier results with human sera Mercaptoethanol as well as heat destroyed the haemolytic function of complement

Mercaptoethanol had a pronounced effect on the neutralizing capacity, even if considerable amounts of undissociated macroglobulin were found in the treated serum by ultracentrifugal analysis

REFERENCES

Bauer D C, Mathies M J & Stavitsky A B Sequences of synthesis of γ 1 macroglobulin and γ 2 globulin antibodies during primary and secondary responses to proteins salmonella antigens and phage J Exp Med *117* 889 907 1963

Berlin B S The nature of antibodies in mice injected with influenza virus vaccine Proc Soc Exptl Biol Med *113* 1013 1016 1963

Deutsch H F & Morton J I Dissociation of human serum macroglobulins Science *125* 600–601 1957

Fulton F & Dumbell K R The serological comparison of strains of influenza virus J Gen Microbiol *3* 97 111 1949

Kjellen L Sterner G & Svedmyr A On the occurrence of adenoviruses in Sweden Acta Paediat Scandinav *46* 164 176 1957

Sterner G & Svartz-Malmberg G Adenovirus neutralizing antibodies in Swedish children Acta Paediat Scandinav *51* 617–620 1962

Svartz-Malmberg G Thermolabile serum factor influencing the neutralization of adenovirus Lancet pp 116–117 Jan 12 1963 a

Svartz Malmberg G Thermolabile serum factor influencing the neutralization of adenovirus Transact Med Microbiol Division of the Swedish Medical Society Acta path et microbiol scandinav *58* 154 1963 b

Svartz-Malmberg G Influence of heat inactivation on the neutralization of type 7 adenovirus by animal sera Acta path et microbiol scandinav *60* 295 296 1964

Svehag S E The formation and properties of poliovirus neutralizing antibody I 19S and 7S antibody formation Differences in kinetics and antigen dose requirement for induction J Exp Med *119* 1 19 1964 a

Svehag S E The formation and properties of poliovirus neutralizing antibody II Normal antibody and early immune antibody of rabbit origin a comparison of biological and physicochemical properties J Exp Med *119* 517 535 1964 b

Svedmyr A Enders J F & Holloway A Complement fixation with the three types of poliomyelitis viruses propagated in tissue culture Am J Hyg 57 60 70 1953

Toussaint A J & Muschel L H Studies on the bacteriophage neutralizing activity of serums J Immunol *89* 27 40 1962

Acta path et microbiol scandinav 66 369–382 1966

The University of Bergen, School of Medicine The Broegelmann Research Laboratory for Microbiology, Bergen, Norway

HETEROGENEITY OF ANTI-γ GLOBULIN FACTORS DETECTED BY PEPSIN DIGESTED HUMAN γG-GLOBULIN

By

JACOB B NATVIG

Received 30.xi 65

Anti γ globulin factors reacting with antigenic determinants of the Fab fragment of human γ globulin revealed by pepsin- or papain- digestion at low pH were demonstrated by *Osterland et al* (21) This "pepsin-agglutinator" was inhibited by pepsin-digested γ globulin (Fab fragments) but not by native, undigested (whole) γ-globulin *Harboe et al* (13) obtained a reaction with whole human γG globulin acting as antibody in an immune precipitate, and *Williams* (28) showed that "pepsin agglutinators" were inhibited by isolated heavy chains of γG-globulin

Corresponding anti γ-globulin factors reacting with hidden antigenic determinants of the light chains of γ globulin have also been demonstrated (4, 27)

"Pepsin agglutinator" activity has been found in γG, γA- and γM globulin classes (13, 21, 24) No individual specificity of the "pepsin agglutinators" has been detected (21, 24)

This paper presents results demonstrating 1 specificity of "pepsin agglutinators" in reaction with Fab fragments of human γG globulin of different Gm character 2 heterogeneity of the Fab fragments probably of the Fd part, of human γG globulin 3 a new type of anti-antibody only detectable by Fab fragments acting as antibody in an immune complex

MATERIALS AND METHODS

Red cells O R_1R_2(CDe/cDE) red cells from a single donor were stored at 4° C in acid-citrate-dextrose solution for a maximum of 5 days

Incomplete anti D anti Gm and normal sera All sera had been used in earlier investigations (15 16 17 18) Gm(a) anti D 3091 and 3311 Gm(b) anti D 3109 and 3500 and Gm(f) anti D 3066 and 3083 were used in undigested form (whole anti D) and after pepsin-digestion (Fab anti D

Sera containing pepsin-agglutinator One hundred sera from patients suffering

Aided in part by a grant from *L Meltzers hogskolefond* Bergen Norway

from rheumatoid arthritis and various other diseases were tested in dilutions 1 2 to 1 64 against red cells sensitized by reference whole anti D or by the corresponding Fab anti D Thirty sera contained 'pepsin agglutinator' in a titre of 8 or higher No activity was detected by the corresponding whole anti-D antibodies However 10 of these sera agglutinated red cells sensitized by whole anti CD Ripley

Anti human gammaglobulin sera Rabbit antiserum (K 980) against whole human serum was used as previously (15) Rabbit anti Fab was obtained by immunizing albino rabbits by intramuscular injection of pepsin digested pooled human γ globulin in complete *Freund's* adjuvant. Specific rabbit anti γG serum (anti Fcγ) was obtained by absorbing anti human γ globulin serum with pepsin digested pooled γ globulin as described by *Fahey* (6) Specific rabbit anti-γA and anti γM sera (Behringwerke AG Marbourg/Lahn, Germany, Op Nr 560 and 374 B respectively) and rabbit anti γM serum (K 43) (provided by Dr M Harboe) were also used

Gammaglobulin Human gammaglobulin 12 per cent solution, batch 80779 was kindly provided by AB Kabi, Stockholm Sweden Six mg/ml of this γ globulin solution or 4 mg/ml of the corresponding Fab fragments did not agglutinate red cells sensitized by the whole or Fab anti D antibodies used

Bence-Jones protein Six urines with Bence-Jones protein 3 of antigenic type K (type I) and 3 of type L (type II)) (see 2 4) were provided by Dr M Harboe Apart from one urine which contained some albumin one homogenous line was obtained when these samples were run in immunoelectrophoresis using rabbit anti human serum Thus no significant amount of other proteins was present Exposure to pepsin and measurement of protein concentration were performed as described below

Pepsin digestion (20 21) Digestion was performed with pepsin (Pepsin 3 X crystallized no P88/061 Sigma Chemical Company, St Louis Missouri, U.S.A.) Gammaglobulin from human sera was prepared by precipitation in 1 33 M $(NH_4)_2SO_4$ (one part of serum and 2 parts of 2 M $(NH_4)_2SO_4$) This precipitation results in fractions which consist primarily of γG globulin (31) Pooled γ globulin from AB Kabi was also digested The ratio of enzyme to protein was 2 100 (w/w) and digestion was carried out for 24 hours at 37° C in 0 1 M acetate buffer providing a pH of 4 1 in the final reaction mixtures Reducing agents were not used The reaction was stopped by raising the pH to 8 0 Following dialysis against saline the protein concentration was measured by a modified *Folin Ciocalteau* method (see 18) For control, γ-globulin was incubated under the same conditions but without pepsin

Agglutination tests (15) Agglutinating activity was tested with red cells sensitized by excess amounts of whole or Fab anti D according to the tube and slide techniques described previously Equal volumes each of sensitized red cells antiserum and saline were mixed

The sensitized red cells gave equally strong and reproducible titres when tested against anti γ globulin and anti Fab sera They were agglutinated by specific anti γG serum but no reaction was obtained with anti γA or anti γM sera Thus the red cells were specifically sensitized with anti D of the γG globulin class (see 3)

That the anti-D sensitized red cells were strongly agglutinated by anti Gm factors which are confined to react with γG globulin, was also in accordance with this view

In inhibition experiments the saline was substituted by an equal volume of the material to be tested Inhibition with γ globulin from anti D sera was performed after absorption with $O R_1R_2$ red cells to remove all anti D activity

Absorption of anti γ-globulin factors Red cells sensitized by whole or Fab anti D antibodies were prepared and tested as described above For each absorption, equal volumes of packed sensitized red cells and antiserum diluted 1 2 were mixed and left at room temperature for 2 hours The supernatant fluids were then tested for activity In controls antiserum was exposed to unsensitized red cells

Reduction by mercaptoethanol Equal volumes of serum and 0 3 M 2 Mercaptoethanol (Fluka AG Buchs Switzerland) in saline were mixed and kept overnight at room temperature The mixtures were then tested for various activities

As in the previous papers (15 17) where this method was employed serum samples treated in a similar way but not exposed to mercaptoethanol served as controls Furthermore known γG and γM antibodies were tested Incomplete anti D (γG antibody) maintained the activity while heterophile antibodies in infectious mononucleosis (γM antibodies) (see 19) were destroyed by the treatment

Immunoelectrophoresis The micromodification using LKB immunoelectrophoresis equipment (LKB produkter AB Stockholm Sweden) was employed The samples were separated in a one per cent agar gel at pH 8 6 applying a current of [illegible] ma for

2 hours at 4° C. Antisera were then added The reactions were observed for at least 2 days

Gm typing Gm typing was performed as previously described (see 15)

EXPERIMENTS AND RESULTS

Testing of Products of Pepsin Digestion

The effect of digestion was tested by agglutination and agglutination inhibition All the γ globulin samples used were either positive for Gm (a), Gm(b) or both The digested products lacked these activities, while controls not exposed to pepsin showed strong inhibition An example with γ globulin of anti-D serum 3091 is shown in Table 1 Furthermore, red cells sensitized with whole Gm(a), Gm(b) or Gm(f) anti D were strongly agglutinated by anti Gm(a), anti-Gm(b) or specific anti-γG serum (anti Fcγ), while red cells sensitized by the corresponding Fab anti D were not agglutinated These findings demonstrated that the Fc fragments were completely destroyed by the pepsin treatment (12, 21)

TABLE 1

Agglutination of Anti Gm(a) in Undigested and Pepsin Digested γ Globulin of Serum 3091

	Reciprocals of dilutions of anti Gm(a) 3071							
	2	4	8	16	32	64	128	256
Test in								
Saline	+++	+++	+++	+++	+++	++	+	—
Digested γ (2 mg/ml)	+++	+++	+++	+++	+++	++	+	—
Undigested γ (3 mg/ml)	—	—	—	—	—	—	—	—

Controls Unsensitized red cells and anti Gm(a) no agglutination
Red cells sensitized by Gm(a) anti D 3091 and saline no agglutination
Sensitized red cells and undigested or pepsin digested γ globulin 3091 no agglutination

In accordance with previous findings (see 18), the Gm(f) inhibiting capacity was not altered after pepsin-digestion, and pepsin-digested Gm(f) anti D still agglutinated anti Gm(f) Pepsin digested anti D gave a well maintained titre in the anti globulin test using anti γ globulin and anti Fab sera (methods as described for whole anti D (15)) The Fab fragments with the antigen binding sites were thus intact

The results obtained in immunoelectrophoresis also showed that the Fc(fast) fragment was destroyed while the Fab(slow) fragment remained

Only samples which fulfilled the above criteria were used Such Fab fragments are bivalent have a sedimentation coefficient of approximately 5S (20), and the configuration and activity of the Fab part of the molecule are then probably optimal (9) These bivalent pepsin-digested fragments are for convenience referred to as Fab antibodies

or Fab γ globulin in this study Since Fab anti D originated from molecules of different Gm type, they were designated by the letter of the Gm type of the corresponding whole anti-D Accordingly pepsin-digested Gm(a) anti D was called Fab(a) anti-D

The protein concentration of such Fab γ-globulin was about 2.3 of that of the corresponding undigested γ-globulin equilibrated to the same volume Since the destroyed Fc fragment makes between 1/2 1 3 of the molecule (23) concentrations of Fab and whole γ globulin were compared in proportions 2 3

Characterization of "Pepsin-Agglutinator" Activity

The different patterns obtained with the 30 "pepsin agglutinator sera are presented in Table 2 Seventeen sera agglutinated red cells sensitized by each type of Fab anti-D, while the other 13 sera showed some selectivity (Table 2) Although equally strong sensitization of red cells was obtained, (see Table 3), 6 sera even showed only monospecific reactions and 5 were bispecific Two sera showed strong activity against one type of Fab anti-D and a very weak reaction (titre 2) with another Fab anti-D, but were called monospecific No agglutination was obtained when the corresponding whole anti-D was used The agglutination was inhibited by Fab γ globulin (1 mg ml) but not by pooled whole γ globulin (3 mg ml) These are typical properties of "pepsin-agglutinators" (21) No inhibition was obtained with pepsin alone or Bence-Jones protein in native form or exposed to pepsin (see later)

Most of the "pepsin agglutinators" were destroyed by mercapto ethanol, but in 4 sera activity remained and thus at least partly resided in other immunoglobulin classes than γM (see 15, 17)

TABLE 2

Activities of 30 Pepsin Agglutinator Sera in Reaction with Fab Anti D A bodies

Type of sera	Number	Red cells sensitized by		
		Fab(a) anti D 3091	Fab(b) anti D 3109	Fab (anti D 30
Polyspecific	17	+++	+++	+
Bispecific	5	+++	−	+
Monospecific	1	+++	−	(
Monospecific	4	−	+++	
"Monospecific"	1	(+)	+++	
Monospecific	2	−		+

+++ strong agglutination (+) very weak agglutination no agglutination
− but not by whole γ but n whole anti D
) Ripley but these actions rs" (see text)

Ten sera contained rheumatoid factors detected by anti CD Ripley These factors were inhibited by pooled native γ-globulin but not by Fab γ-globulin and were thus distinguishable from the "pepsin-agglutinators" They were all destroyed by mercaptoethanol indicating that they were γM-globulins (see 15, 17)

Agglutination Experiments

One polyspecific, 3 bispecific and 6 monospecific "pepsin agglutinator" sera were further tested against red cells sensitized by each of 6 Fab anti D antibodies respectively (Table 3) Anti Gm(f) and rabbit antiglobulin serum were included as controls Since red cells sensitized by the various Fab anti-D antibodies did not agglutinate anti Gm(b), this anti Gm serum was used as negative control

TABLE 3

Titres of Individual Pepsin Agglutinator Sera Tested with Selected Fab Anti D Antibodies

Serum	Red cells sensitized by					
	Fab(a) anti D		Fab(b) anti D		Fab(f) anti D	
	3091	3311	3109	3500	3083	3066
4090	64	64	64	32	64	64
4110, 4143 4148	16 64	16 64	<2	<2	16 32	8 32
4156	16	16	<2	<2	2	2
4087, 4114 4117	<2	<2	16–64	16–64	<2	2 8
4119	<2	<2	<2	<2	64	<2
3731	<2	<2	<2	<2	16	16
Controls						
Anti Gm(f) A J	8	8	8	8	64	64
Anti Gm(b) 2535	<2	<2	<2	<2	<2	<2
Antiglobulin serum K 980	1024	1024	1024	512	1024	1024

e respective

The "pepsin-agglutinator" 4156 reacted with each of the 2 Fab(a) anti D antibodies No reaction with 2 Fab(b) anti-D and very weak reactions with the 2 Fab(f) anti D antibodies was obtained Three "pepsin-agglutinators" (4087, 4114, 4117) reacted with 2 Fab(b) anti D antibodies The 2 Fab(a) anti D or Fab(f) anti-D 3083 did not detect any activity in these 3 sera Weak reactions were demonstrated with Fab(f) anti D 3066, but this anti D contained a small amount of *Gm(b) molecules (15 16) Since anti Gm(b) did not agglutinate* red cells sensitized by the whole anti-D 3066 it is probable that the "pepsin-agglutinators" are more sensitive in agglutination reaction

The "pepsin-agglutinator" 3731 agglutinated red cells sensitized by the 2 Fab(f) anti D antibodies respectively while "pepsin agglutinator" 4119 agglutinated only red cells sensitized by Fab(f) anti D 3083 An

equally strong agglutination of red cells sensitized by the 2 Fab(f) anti-D antibodies was obtained with anti-Gm(f) A. J This anti Gm(f) also showed weak "pepsin-agglutinator" reactions with Fab(a) and Fab(b) anti-D

"Pepsin-agglutinators" 4110, 4143 and 4148 reacted with the 2 Fab (a) and the 2 Fab(f) anti-D antibodies, while no reaction was obtained when the 2 Fab(b) anti-D antibodies were used "Pepsin-agglutinator" 4090 showed a polyspecific reaction comparable to that of the antiglobulin serum

No prozones were observed in "pepsin-agglutinator" reactions There was no difference in the agglutination whether unheated or heat inactivated (56° C for 1/2 hour) sera were used

Absorption Experiments

The monospecific "pepsin-agglutinator" sera 4156, 4087 and 4119 were absorbed by red cells sensitized by Fab(a), Fab(b) and Fab(f) anti D respectively (Table 4) The activity of a serum was only removed by the same type of sensitized red cells as were agglutinated by the respective serum, but not by the 2 other types of sensitized red cells These results further demonstrated that the "pepsin-agglutinators" were of different specificities

Red cells sensitized by the respective whole anti-D antibodies did not absorb the "pepsin-agglutinators"

Crossabsorptions of polyspecific "pepsin-agglutinator" sera were not undertaken because of the great quantity of Fab anti D required

TABLE 4

Titres of Pepsin Agglutinator Sera before and after Various Absorptions

Serum	Absorption with red cells			
	unsensitized	sensitized by		
		Fab(a) anti D 3091	*Fab(b)* anti D 3109	*Fab(f)* anti D 3093
4156	8	<2	16	8
4087	32	32	<2	32
4119	32	32	32	2

Agglutinating activity of the "pepsin agglutinators" 4156 4087 and 4119 were tested with red cells sensitized by Fab(a) Fab(b) and Fab(f) anti D respectively
Controls Unsensitized red cells and the respective sera no agglutination
Sensitized red cells and saline no agglutination

Agglutination-Inhibition Experiments

The 30 "pepsin-agglutinator" sera were tested by agglutination inhibition Fab γ-globulin (1 mg ml), from individual anti D sera absorbed of anti-D activity was added to twofold dilutions of the "pepsin agglutinator" sera Fab and whole pooled γ-globulin were included as controls

and gave similar results as described above Two "pepsin agglutinators" (3731 and 4119) were inhibited by the 5 Fab γ-globulin fractions obtained from Gm (a+x—b+f+) or Gm (a—x—b+f+) sera (Table 5) Fab γ-globulin 3091 from a Gm (a+x+b—f—) serum did not inhibit agglutination, even not in a concentration of 2 mg ml In these experiments the 2 "pepsin-agglutinators" gave similar results as anti-Gm(f) and they possibly indicated factors close to Gm(f)

TABLE 5

Titres of Pepsin Agglutinator Sera and Anti Gm(f) Serum in Whole γ Globulin (1.5 mg/ml) and Fab γ Globulin (1 mg/ml)

	Gm type of respective whole γ glob	Pepsin Agglutinator" 3731	4119	4090	Anti Gm(f) AJ
Test in					
Saline	—	16	64	64	64
Whole γ globulin					
pooled	Gm(a+x+b+f+)	16	64	64	<2
Fab γ globulin					
3091	Gm(a+x+b—f—)	16	64	<2	64
3311	Gm(a+x—b+f+)	<2	<2	<2	<2
3066 3083 3109 3500	Gm(a—x—b+f+)	<2	<2	<2	<2
pooled	Gm(a+x+b+f+)	<2	<2	<2	<2

Tests were performed with red cells sensitized by Fab(f) anti D 3083, except for anti Gm(f) where whole Gm(f) anti D 3083 was used
Fab γ globulin from anti D sera was absorbed of anti D activity before use in inhibition
Controls Unsensitized red cells and the respective pepsin agglutinator sera or anti Gm(f) serum no agglutination Sensitized red cells and saline no agglutination Sensitized red cells and whole or Fab γ globulin no agglutination

As Fab anti D 3109, 3311 or 3500 did not detect the activity in sera 3731 and 4119, γ-globulin of these sera contained a factor not present on the anti D molecules of the same serum

These results further demonstrated the heterogeneity of the Fab fragment of γG-globulin

Each of the other "pepsin agglutinators" was inhibited by each of the 6 individual Fab γ-globulin fractions tested, irrespective of the specificities of the "pepsin agglutinators' and of inhibiting effect of the fractions in other reactions

The inhibiting effect of Fab γ-globulin in two fold dilutions (from 1 mg ml) was tested against 15 "pepsin-agglutinator" sera diluted 1 2 (8 to 32 agglutinating units) The sera included all the different types of specificities described above Depending on the number of agglutinating units in a given serum, inhibition was obtained with Fab γ globulin in concentrations from 0 25 to 0 03 mg ml Very similar effects were obtained with the 6 individual Fab γ-globulin samples and Fab pooled γ globulin in a given serum

Inhibition experiments with *Bence-Jones* proteins were then per-

formed Such proteins consist only of light chains of γ-globulin (2) An equal amount of *Bence-Jones* protein (1 mg ml) was added to each of twofold dilutions of 15 "pepsin-agglutinator" sera representing the different types of specificities described In order to exclude possible heterogeneity of *Bence-Jones* proteins in these reactions each 'pepsin agglutinator" serum was tested against 6 different proteins There was no inhibition compared to agglutination in saline In controls the 'pepsin agglutinators' were completely inhibited by Fab γ-globulin *Bence-Jones* proteins were exposed to pepsin in a similar way as described for whole γ-globulin No inhibition was obtained with these pepsin treated *Bence Jones* proteins even in a concentration of 2 mg/ml No agglutination test was available in which the inhibiting capacity of *Bence-Jones* proteins could be tested, but the protein showed good reaction in immunoelectrophoresis Furthermore, all the *Bence-Jones* fractions used lacked Gm(a), Gm(b) and Gm(f) inhibiting capacity demonstrating that no other γG globulin fractions were present

The lack of inhibition of "pepsin agglutinators' by light chains from *Bence-Jones* protein indicated that the "pepsin-agglutinators" were directed against determinants of the Fd fragments of the heavy chain, or determinants of the tertiary structure between the heavy and light chains

TABLE 6

Titre of Serum 4124 before and after Various Absorptions

	Unabsorbed control	Absorption with red cells unsensitized	sensitized by whole Gm(f) anti D 3083	sensitized by Fab(f) anti D 3083
Test in				
Saline	32	16	16	<2
Whole γ glob pooled	32	16	16	<2
Whole γ glob 3083	32	16	16	2
Fab γ glob pooled	32	16	16	2
Fab γ glob 3083	32	16	16	<2

Test system and controls as in Table 5
The γ globulin 3083 was absorbed of anti D activity before use in inhibition experiment The fractions used in inhibition did not agglutinate sensitized red cells

Characterization of Agglutinating Activity in Serum 4124

Serum 4124 agglutinated red cells sensitized by Fab anti D 3083 to a titre of 32 No agglutination occurred when whole Gm(a), Gm(b) or Gm(f) anti-D antibodies were used Fab(a) and Fab(b) anti D detected weak pepsin agglutinators" in this serum, and whole anti CD Ripley detected a rheumatoid factor inhibited by whole but not by Fab γ-globulin Judged from the loss of activity by mercaptoethanol treatment each of these activities of serum 4124 belonged to the γM globulin class (see 17)

The activity with red cells sensitized by Fab(f) anti D was of an

anti antibody type since no inhibition of agglutination was obtained with whole or Fab pooled γ globulin, or such γ globulin from serum of the donor of the Fab(f) anti D used for the detection of the agglutinating activity of serum 4124 (Table 6) No inhibition was obtained with each of 6 different *Bence Jones* proteins

Agglutination was also obtained when serum 4124, Fab(f) anti D 3083 and unsensitized red cells were mixed in the same way as previously described with anti antibodies (17) The activity was not influenced when serum 4124 was absorbed with red cells sensitized by whole Gm(f) anti D 3083 which easily removed anti antibody 55 Agglutination was still not inhibited by whole or Fab γ globulin On the other hand red cells sensitized by Fab(f) anti D 3083 easily removed the anti antibody activity of serum 4124 This serum did not agglutinate unsensitized red cells It made no difference to the agglutination of red cells sensitized by Fab anti D whether unheated or heat inactivated sera were used

TABLE 7

Properties of Various Anti γ Globulin Factors in Agglutination and Agglutination Inhibition Tests

	Agglutination test with*		Inhibition test with†	
	whole anti D	Fab anti D	whole γ glob	Fab γ glob
Anti Gm(a x b)	+	—	+	
Anti Gm(f) anti Inv	+	+	+	+
Anti antibody§	+	+	—	
Pepsin agglutinator		+	—	+
Serum 4124		+	—	—

\+ agglutination no agglutination
† + inhibition — no inhibition
§ See Natvig (19)

Table 7 shows the reaction patterns of this new type of factor which differs from other anti γ globulin factors detected by isologous γG antibodies This special factor is provisionally called Fab anti antibody since it showed the following criteria 1 It reacted only with hidden antigenic determinants revealed by pepsin digestion of γ globulin 2 It reacted only with these Fab fragments acting as antibody in an immune complex

Gm Types

The 30 pepsin agglutinator sera were Gm typed Eight sera were Gm(a+x+b+f+) 6 were Gm(a+x—b+f+) 13 were Gm(a x—b +f+) one was Gm(a+x+b—f—) and 2 were Gm(a+x—b—f—)

The specificity of the pepsin agglutinators and the Gm types of the respective sera were compared In one case a monospecific pepsin agglutinator reacted with Fab anti D of γ globulin molecules with a

contribute to elucidating whether this fragment is a separate chain in the γG-globulin molecule as suggested by findings of *Franklin* (8) and others (see 7)

One anti-γ-globulin factor reacted only with pepsin-digested γ-globulin acting as antibody in an immune complex, thus behaving as an anti-antibody (see 17) The reaction of this new type of anti-γ-globulin factor, provisionally called "Fab anti-antibody" was restricted to Fab (f) anti-D This was in accordance with the previously demonstrated predominance of Gm(f) antibodies to detect anti-antibodies (17)

This "Fab anti-antibody" differed from the rabbit and human anti-antibodies reacting with Fab fragments, as these anti-antibodies also reacted with whole γG-immune complexes (see 19) The demonstration of one anti-antibody which reacted with the Fc and not with the Fab fragments (13) further indicated the existence of various classes of anti-antibodies.

Different antigenic determinants are probably involved in anti-antibody and "pepsin-agglutinator" reactions "Pepsin-agglutinators" are inhibited by "native" Fab γ-globulin while anti antibodies are not inhibited The "pepsin-agglutinators" are furthermore not absorbed by immune complexes (agglutinates) with whole anti-D which easily react with the anti-antibodies On the other hand, some similarities may exist since *Harboe & al* (13) showed that immune precipitates with whole γG-antibodies absorbed "pepsin-agglutinators" *Williams & Kunkel* (29) suggested that the sites on the γG-globulin molecules involved by "pepsin-agglutinators" and anti-antibodies are close This is consistent with own findings indicating that specificities of both anti antibodies (19) and "pepsin-agglutinators" are related to determinants of the Fd fragment

The distribution of Gm types in the "pepsin-agglutinator" sera was Gm(a) 56 7 per cent, Gm(x) 30 0 per cent and Gm(b) or Gm(f) 90 0 per cent This is in accordance with the known frequencies of these Gm types, except the frequency of Gm(x) which is somewhat lower (see 17) No strict correlation could be drawn between the "pepsin agglutinator" specificity and the Gm types of the γG globulin in the same serum

SUMMARY

1 Thirty out of 100 sera contained anti γ-globulin factors against red cells sensitized by pepsin-digested incomplete anti-D Using selected pepsin-digested anti-D antibodies, individual specificity of these anti γ-globulin factors ("pepsin-agglutinators") was demonstrated

2 The individual specificity of single "pepsin-agglutinators" indicated heterogeneity of the Fab-fragments of human γG-globulin probably of the Fd part

3 The specificities of 2 "pepsin-agglutinators" indicated the presence of immunoglobulin factors, probably close to Gm(f)

4 A new type of anti antibody which reacted only with pepsin digested Gm(f) anti D in immune complexes was demonstrated

REFERENCES

1 *Ballieux R E Bernier G M Tominaga K & Putnam F W* Gamma globulin antigenic types defined by heavy chain determinants Science *145* 168–170 1964

2 *Berggard I & Edelman G M* Normal counterparts to Bence-Jones proteins free L polypeptide chains of human γ globulin Proc nat Acad Sci (Wash) *49* 330–337 1963

3 *Dodd B F & Wilkinson P C* A study of the distribution of incomplete rhesus antibodies among the serum immunoglobulin fractions J exp Med *120* 45–55 1964

4 *Epstein W V & Gross D* Naturally occurring human antibody reacting with Bence Jones proteins J exp Med *120* 733 745 1964

5 *Fahey J L* Contribution of γ globulin subunits to electrophoretic heterogeneity identification of a distinctive group of 6 6S γ myeloma proteins Immunochemistry *1* 121 131 1964

6 *Fahey J L & McLaughlin C* Preparation of antisera specific for 6 6 S γ globulins β_{2A} globulins γ_1 macroglobulins and for type I and II common γ globulin determinants J Immunol *91* 484–497 1963

7 *Frangione B & Franklin E C* Structural studies of human immunoglobulins Differences in the Fd fragments of the heavy chains of G myeloma proteins J exp Med *1??* 1 10 1965

8 *Franklin E C* Structural studies of human 7S γ globulin (G immunoglobulin) Further observations of a naturally occurring protein related to the crystallizable (fast) fragment J exp Med *120* 691 709 1964

9 *Greenbury C L, Moore D H & Nunn L A C* The reaction with red cells of 7S rabbit antibody its sub-units and their recombinants Immunology *8* 420–431 1965

10 *Grey H M & Kunkel H G* H chain subgroups of myeloma proteins and normal 7S γ globulin J exp Med *120* 253 266 1964

11 *Harboe M* Pepsin splitting of γM globulins and high molecular weight antibodies Scand J clin Lab Invest *17* (suppl 84) 233 245 1965

12 *Harboe M Osterland C K & Kunkel H G* Localization of two genetic factors to different areas of γ globulin molecules Science *13?* 9?9 980 196?

13 *Harboe M Rau B & Aho K* Properties of various anti γ globulin factors in human sera J exp Med *121* 503 519 1965

14 *Kunkel H C Allen J C Grey H M Mårtensson L & Grubb R* A relationship between the H chain groups of 7S γ globulin and the Gm system Nature (Lond) *203* 413–414 1964

15 *Natvig J B* Incomplete anti D antibody with changed Gm specificity Acta path et microbiol scandinav *65* 467 479 1965

16 *Natvig J B* Quantitative determinations of incomplete anti D antibodies in reaction with anti Gm factors Acta path et micr biol scandinav *65* 570–580 1965

17 *Natvig J B* Specificity of anti antibodies in human sera Acta path et microbiol scandinav *65* 559 569 1965

18 *Natvig J B* Heterogeneity of anti antibodies in individual human sera Acta path et microb l scandinav *66* 188 196 1966

19 *Natvi* [illegible]

20 *Nisor* [illegible]

21 *Oster* [illegible] sera revealed by enzymatic splitting of anti [illegible] antibodies Vox Sang *8* 133–152 1963

22 *Polmar S H & Steinberg A G* Dependence of a Gm(?) antigen on the quaternary structure of human gamma globulin Science *145* 9?8 9?9 1964

23 *Porter, R R* The hydrolysis of rabbit γ globulin and antibodies with crystalline papain Biochem J *73* 119 126 1959
24 *Schoenfeld L S & Epstein W V* Macroglobulin rheumatoid factors directed toward buried antigenic sites of human γ globulins Vox Sang *10* 482 492 1965
25 *Steinberg A G & Polmar, St H* The relation of the S and F fragments and the H and L chains of gamma globulin to the Gm groups Vox Sang *10* 361 370 1965
26 *Terry W D & Fahey J L* Subclasses of human γ_2 globulin based on differences in the heavy polypeptide chains Science *146* 400–401 1964
27 *Williams R C* Heterogeneity of L-chain sites on Bence-Jones proteins reacting with anti γ globulin factors Proc nat Acad Sci (Wash) *52* 60–64 1964
28 *Williams R C* Reaction of human and rabbit anti gamma globulin factors with heavy and light chains of gamma globulin Arthr and Rheum *7* 368 378 1964
29 *Williams R C & Kunkel H G* Observations on antigamma globulin factors in human sera and antibodies against autologous gamma globulins produced in rabbits Ann N Y Acad Sci *124* 860 864 1965

Acta path. et microbiol. scandinav. 66, 383–395, 1966

The University of Bergen School of Medicine. The Broegelmann Research Laboratory for Microbiology, Bergen, Norway

REACTION OF HUMAN ANTI-ANTIBODIES WITH INCOMPLETE ANTI-D AND ITS SUB-UNITS

By

JACOB B. NATVIG

Received 30.xi.65

Individual specificity of anti-antibodies in different sera in reaction with γG-globulin of various Gm types was recently demonstrated (20). Furthermore, heterogeneity of anti-antibodies in single sera was found (21).

Anti-γ-globulin factors detected by pepsin-digested γG globulin, for convenience called "pepsin-agglutinators", also showed individual specificity (22). They indicated heterogenic determinants probably on the Fd part of the Fab fragment.

The "pepsin agglutinators" were designated by the letter of the Gm specificity of the anti-D which after pepsin-digestion was used for their detection (22).

A similar notation will be used to indicate the individual specificity of anti-antibodies. Thus, anti-antibody(f) is an anti-antibody detected by Gm(f) anti-D. Polyspecific sera may for example be designated as anti-antibody(a, f) or by the 2 specificities separate if they are distinguishable by absorption (anti-antibody(a) and anti-antibody(f)).

The use of pepsin-digested γG-globulin (Fab fragments) gives a valuable tool for studies of anti-antibodies. Previous investigations in this field have been contradictory. *Fudenberg et al.* (4) showed that rabbit anti antibodies reacted with Fab fragments of the antibody in an immune complex. *Harboe et al.* (8) found that a human anti-antibody reacted with determinants of the Fc fragment.

"Pepsin-agglutinators" are frequent in anti-antibody sera (8) but the further relation between the 2 types of anti-γ-globulin factors in individual sera is not known.

This paper presents observations on: 1. the specificity of "pepsin-agglutinators" in anti-antibody sera. 2. the specificity of anti-antibodies in reaction with red cells sensitized by whole or pepsin-digested anti D of various Gm types.

Aided in part by a grant from *L. Meltzers høyskolefond*, Bergen, Norway.

MATERIALS AND METHODS

Red cells O R_1R_2 (CDe, cDE) red cells from a single donor were used (18)

Anti antibody sera Nine out of the 14 sera Gm typed and used in a previous study (20) were employed Another anti antibody serum (700), briefly mentioned earlier (21) was also included The sera contained anti γG globulin factors with definite anti antibody activity, and represented the different specificities previously described (20)

Incomplete anti D anti-Gm pepsin-agglutinator and normal sera All sera were used and characterized in earlier investigations (18 19 22) The anti D antibodies were γG globulins and were used in undigested form (whole anti D) and after pepsin digestion (Fab anti D)

According to the previously described notation (22) Fab Gm(a) Gm(b) or Gm(f) anti D antibodies were called Fab(a) Fab(b) and Fab(f) anti D respectively

Anti human gammaglobulin sera Only rabbit antisera previously used were employed (18, 22)

Gammaglobulin Human gammaglobulin 12 per cent solution batch 80779 was kindly provided by AB Kabi Stockholm Sweden The γ globulin and fragments hereof were denatured by heating the solution at 63° C for 10 min (1 2) The native or denatured γ globulin or γ-globulin fragments obtained from enzyme digestion (see later) did not agglutinate the sensitized red cells used in this study

Enzyme digestion of proteins (24) The pepsin digested material prepared earlier was employed (22) The digestion was carried out at pH 4 1 using a ratio of enzyme to protein of 2 100 (w/w) Previous extensive testing (22) of the material obtained showed that the Fab fragments were intact while the Fc fragments of γ globulin were completely destroyed

Pooled γ globulin was digested by papain (papain 2 × crystallized lot no 938 1810 Sigma Chemical Co St Louis, Missouri U S A) in 0 1 M phosphate buffer at pH 7 4 for 24 hours at 37° C The ratio of enzyme to protein was 1 100 (w/w) and the reaction mixture was made 0 01 M in cysteine and 0 002 M in ethylendiamine tetraacetate

In immunoelectrophoresis the papain-digested products showed characteristic Fab(slow) and Fc(fast) lines crossing each other without interference The preparation had maintained Gm inhibiting capacity (Gm(a) Gm(b) Gm(f)) further showing that the antigenic determinants of the Fc and Fab fragments were intact

Similarly treated γ globulin samples not exposed to enzyme served as controls

As previously (22) Fab γ globulin and Fab antibodies are employed as notations for pepsin-digested products Products of papain digestion will be further specified Fab fragments obtained after pepsin and papain digestion respectively are however probably very similar (6 15 24)

Agglutination tests Agglutinating activity was tested by the tube and slide techniques described previously (18) Strongly sensitized red cells tested by anti globulin sera were used

In inhibition experiments the saline was substituted by an equal volume of the material to be tested Inhibition with anti D sera was performed after al[illegible] of all anti D activity (20)

Absorption experiments Absorption with red cells sensitized by whole or Fab anti D (γG antibodies) was performed as described elsewhere (22)

Absorption with γM antibodies was performed using sheep red cells agglutinated by heterophile antibodies in sera of patients suffering from infectious mononucleosis Five individual heat inactivated sera (56° C for 30 min) with a min[illegible] antibody titre of 2048 and a typical absorption pattern (sec 13) were selected That the antibodies were γM globulins was confirmed by mercaptoethanol treatment and ultracentrifugation (20)

Sheep red cells were sensitized with an excess of antibody (50 agglutinating units (18)) at room temperature for 1 hour The red cells were thoroughly washed [illegible] times Equal volumes of packed sensitized red cells and anti antibody serum diluted 1 2 were mixed and left at room temperature for 2 hours The [illegible] fluids were tested against anti D sensitized red cells In controls the sera were exposed to unsensitized red cells

Reduction by mercaptoethanol This was performed as previously [illegible] controls with known γG and γM antibodies (22

Immunoelectrophoresis This test was performed by the microtechnique as previously described (22)

EXPERIMENTS AND RESULTS

Coexistence of "Pepsin Agglutinators", Anti-Antibodies and Anti Gm(a) in Individual Sera

The 10 anti-antibody sera were tested against red cells sensitized by whole Gm(a), Gm(b) and Gm(f) anti D and the corresponding Fab anti D antibodies The 10 sera were also exhausted of anti antibody activity by absorption employing whole anti-D corresponding to each of the anti antibody specificities in a given serum Sera exposed to unsensitized red cells served as controls and gave similar results as when untreated sera were tested All the tests were run in saline, pooled whole γ globulin (6 mg ml) and Fab γ-globulin (4 mg ml)

The anti antibodies, "pepsin-agglutinators" and anti Gm factors were clearly distinguishable by their patterns in agglutination inhibition tests When inhibition was obtained it was always complete (titre < 2) The agglutination obtained in series not showing inhibition never differed more than one titre value from controls in saline Anti Gm(a) was inhibited by whole, but not by Fab γ globulin (reaction pattern designated (g)) "Pepsin agglutinators" were inhibited by Fab, but not by whole γ-globulin (reaction pattern designated (p)) and anti-antibodies were not inhibited either by Fab or whole γ globulin (reaction pattern designated (a))

The unabsorbed anti antibody sera showed the same type of reactions against red cells sensitized by various whole anti D antibodies as in previous studies (20, 21) (Tables 1 and 2) A new sample of anti antibody serum 648 gave a weak reaction detected by Gm(a) anti D (21) Anti-antibody serum 700 of Gm type Gm(a—x—b+f+), contained an isospecific anti-Gm(a), a heterospecific rheumatoid factor and anti antibody(f), and thus had the same combination of anti-γ globulin factors as serum 355 (cfr 20, Table 2)

The further results obtained with anti antibody(f) sera are shown in Table 1 In all sera anti antibody activity was detected not only by whole anti D but also by the corresponding Fab anti-D Thus in serum 55 anti antibody activity was demonstrated by whole Gm(f) anti D and Fab(f) anti-D while no anti antibody was found by using whole or Fab anti-D of the other specificities However "pepsin agglutinators" reacted with Fab anti D antibodies which did not detect any anti antibody After absorption employing whole anti D, anti-antibody activity was no longer detected by either whole or Fab anti D However, in each absorbed serum a "new" "pepsin agglutinator", reacting with the Fab anti D which detected anti-antibody activity in the unabsorbed serum was revealed Absorption of anti Gm(a) was not performed since previous investigations (see 22) showed that this factor does not react with Fab anti-D

TABLE 1

Titres of Anti Antibody(f) Sera against Red Cells Sensitized by Whole or Fab Anti D before and after Absorption of Anti Antibody Activity

Serum		Test with red cells sensitized by Gm(a) anti D 3091 whole	Fab	Gm(b) anti D 3109 whole	Fab	Gm(f) anti D 3083 whole	Fab
55	ctr	<2	16 (p)	<2	16 (p)	64 (a)	128 (a)
	abs	<2	16 (p)	<2	8 (p)	<2	32 (p)
139	ctr	<2	32 (p)	<2	16 (p)	32 (a)	32 (a)
	abs	<2	32 (p)	<2	8 (p)	<2	32 (p)
355	ctr	512 (g)	64 (p)	<2	128 (p)	32 (a)	64 (a)
	abs	256 (g)	64 (p)	<2	128 (p)	<2	64 (p)
576	ctr	<2	32 (p)	<2	16 (p)	64 (a)	64 (a)
	abs	<2	32 (p)	<2	16 (p)	<2	16 (p)
647	ctr	<2	16 (p)	<2	<2	32 (a)	32 (a)
	abs	<2	32 (p)	<2	<2	<2	32 (p)
700	ctr	256 (g)	16 (p)	<2	16 (p)	16 (a)	16 (a)
	abs	256 (g)	16 (p)	<2	16 (p)	<2	4 (p)

ctr control exposed to unsensitized red cells abs absorbed
Titre of antiglobulin serum (K 980) against each type of sensitized red cells as in Table 2 Each reaction was characterized in inhibition using whole γ globulin (6 mg/ml) and Fab γ globulin (4 mg/ml) The typical reaction patterns (see text) are designated a anti antibody g anti Gm and p pepsin agglutinator
Controls Unsensitized red cells and respective anti antibody sera no agglutination
Sensitized red cells and saline whole or Fab γ globulin no agglutination

TABLE 2

Titres of Polyspecific Anti Antibody Sera against Red Cells Sensitized by Whole or Fab Anti D before and after Absorption of Anti Antibody Activity

Serum		Test with red cells sensitized by Gm(a) anti D 3091 whole	Fab	Gm(b) anti D 3109 whole	Fab	Gm(f) anti D 3083 whole	Fab
Anti antibody (a f) serum							
356	ctr	32 (a)	64 (a)	<2	<2	16 (a)	32 (a)
	abs	<2	16 (p)	<2	<2	<2	16 (p)
648	ctr	4 (a)	8 (a)	<2	16 (p)	64 (a)	64 (a)
	abs	<2	8 (p)	<2	16 (p)	<2	16 (p)
649	ctr	16 (a)	16 (a)	<2	<2	16 (a)	16 (a)
	abs	<2	<2	<2	<2	<2	<2
Anti antibody (a b f) serum							
141	ctr	64 (a)	64 (a)	8 (a)	4 (a)	64 (a)	64 (a)
	abs	<2	16 (p)	<2	2 (p)	<2	16 (p)
Antiglobulin serum K 980		1024	512	512	512	1024	512

A new sample of serum 648 had a partly changed specificity (21) Each reaction was characterized in inhibition using whole γ globulin (6 mg/ml) and Fab γ globulin

Table 2 shows the results obtained with polyspecific anti-antibodies In anti-antibody(a,f) sera, anti-antibody activity was detected both by Gm(a) and Gm(f) anti D and the 2 corresponding Fab anti-D antibodies After absorptions using whole anti-D, no anti-antibody activity was present, while "pepsin-agglutinators" reacting with Fab(a) and Fab(f) anti-D were found in 2 sera Similar results were obtained in anti antibody(a b,f) serum 141 although the reaction with Fab(b) anti-D was very weak Serum 649 did not contain any "pepsin agglutinator"

Each anti-antibody was detected by one type of whole anti-D and by its corresponding Fab fragment Only one serum lacked "pepsin-agglutinators" Apart from this serum one "pepsin-agglutinator" always reacted with the Fab fragment of the anti-D which detected an anti-antibody in the respective serum Thus, if anti antibody was detected by Gm(f) anti D, "pepsin agglutinator"(f) was present

Seven sera contained "pepsin-agglutinators" against all 3 types of Fab anti-D, although some of the "pepsin agglutinator"(b) activities were very weak Two sera (356 and 647) contained only "pepsin-agglutinators" restricted to Fab(a) and Fab(f) anti-D No anti-antibody serum showed only one monospecific "pepsin agglutinator" Such sera thus exhibited more polyspecific reactions than some "pepsin-agglutinator" sera previously described (22) The experiments showed that the "pepsin agglutinators" and anti antibodies were closely related, and indicated that anti antibodies reacted with immune complexes containing Fab anti-D

Mercaptoethanol destroyed each of the activities detected by whole and Fab anti-D, indicating that both "pepsin-agglutinators" and anti antibodies in these sera were γM-globulins

Further Tests of Anti Antibodies using Whole or Fab Anti-D

Four anti-antibody sera representing the different specificities were tested in absorptions with whole and Fab anti-D and agglutination inhibition with whole and Fab γ globulin As demonstrated above, serum 55 contained typical "pepsin agglutinators" reacting with Fab (a) and Fab(b) anti-D (Table 3) In contrast, the reaction using Fab(f) anti D was of the anti-antibody type, not inhibited by either whole or Fab γ globulin Anti-antibody(f) serum 55 absorbed of anti-antibody showed a typical "pepsin-agglutinator" reaction with Fab(f) anti D Red cells sensitized by Fab(a), Fab(b) and Fab(f) anti D respectively absorbed the homologous "pepsin-agglutinators" In addition red cells sensitized by Fab(f) anti D also removed anti-antibody(f) 55 Similar results were obtained by identical absorption procedures with anti antibody(f) serum 576

According to previous findings the reactions of serum 356 with Fab (a) and Fab(f) anti-D were both of anti-antibody type as when using

the corresponding whole anti-D antibodies (Table 4) Absorptions with red cells sensitized by whole Gm(a) and Gm(f) anti D respectively removed the anti antibody completely The activities detected by Fab (a) and Fab(f) anti-D then changed to typical "pepsin-agglutinator reactions, easily inhibited by Fab γ-globulin Absorption using Fab(a) and Fab(f) anti-D removed "pepsin agglutinator" and the corresponding anti antibody activity detected by whole anti D A similar pattern was obtained with anti antibody (a, b, f) serum 141 in reactions with Gm(a) and Gm(f) anti D or the Fab fragments hereof The reactions with Gm(b) anti D were rather weak and the results inconclusive

TABLE 3

Titres of Anti Antibody(f) Serum 55 in Saline (A) in Whole γ Globulin (B) and in Fab γ Globulin (C) before and after Various Absorptions

		Test with red cells sensitized by whole					
		Gm(a) anti D	Gm(b) anti D	Gm(f) anti D	Fab(a) anti D	Fab(b) anti D	Fab(f) anti D
Absorption with red cells							
unsensitized	A	<2	<2	64	16	16	128
	B			64	16	16	128
	C			64	<2	<2	128
sensitized by whole anti D	A	<2	<2	<2	16	8	32
	B				8	8	32
	C				<2	<2	<2
sensitized by Fab anti D	A	<2	<2	<2	<2	<2	<2

For absorptions with sensitized red cells see text
Controls as in Table 1

TABLE 4

Titres of Anti Antibody (a f) Serum 356 in Saline (A) in Whole γ Globulin (B) and in Fab γ Globulin (C) before and after Various Absorptions

		Test with red cells sensitized by whole					
		Gm(a) anti D	Gm(b) anti D	Gm(f) anti D	Fab(a) anti D	Fab(b) anti D	Fab(f) anti D
Absorption with red cells							
unsensitized	A	32	<2	16	32	<2	32
	B	32		16	32		32
	C	32		16	32		32
sensitized by whole anti D	A	<2	<2	<2	16	<2	16
	B				16		16
	C				<2		<2
sensitized by Fab anti D	A	<2	<2	<2	<2	<2	<2

For absorptions with sensitized red cells see text
Controls as in Table 1

These findings further showed that anti antibody activity in the present sera was detected by Fab anti D

In the agglutination experiments anti antibody reactions were only obtained with the Fab fragment of the anti D used to detect the anti antibody, indicating that their specificity was related to heterogenic determinants of the Fab fragment

This was sustained by the further results of absorptions using Fab (a) Fab(b) and Fab(f) anti D The anti antibody (f) 55 was only removed by Fab(f) anti D (Table 5) and absorptions on anti antibody (f) serum 576 gave comparable results

TABLE 5

Titres of Anti Antibody Sera 55 and 3o6 before and after Absorptions Using Fab Anti D of Different Specificities

	Serum 55 Test with		Serum 3o6 Test with			
	whole Gm(f) anti D	Fab(f) anti D	whole Gm(a) anti D	Fab(a) anti D	whole Gm(f) anti D	Fab(f) anti D
Absorption with red cells						
unsensitized	32	32	32	32	16	16
sensitized by						
Fab(a) anti D	16	32	<2	<2	16	16
Fab(b) anti D	32	32	16	16	16	16
Fab(f) anti D	<2	<2	16	16	<2	<2

Each of the detected activities was of anti antibody type Controls as in Table 1

Anti antibody (a f) serum 3o6 was then absorbed and the activities were tested with whole Gm(a) and Gm(f) anti D and with the corresponding Fab fragments These absorptions also demonstrated the strict specificity of anti antibodies for determinants of the Fab fragments Furthermore a crossabsorption was obtained similar to that demonstrated previously (cfr 21 Table 2) by using whole anti D

Test with γM Antibodies

Since γM antibodies give no subagglutinating sensitization of red cells (27) only absorption experiments were performed

Each of the 10 anti antibody sera was absorbed by each of 5 batches of sheep red cells sensitized by 5 individual γM antibodies respectively Activity in a given serum was tested using Gm(a) Gm(b) and Gm(f) anti D No anti antibody was absorbed by any of the red cells sensitized by γM antibodies The titres of the absorbed sera and controls never differed more than one step After the absorption procedures each of the activities was still of anti antibody nature Immune complexes containing γM antibodies thus did not react with anti antibodies

the corresponding whole anti-D antibodies (Table 4) Absorptions with red cells sensitized by whole Gm(a) and Gm(f) anti-D respectively removed the anti antibody completely The activities detected by Fab (a) and Fab(f) anti-D then changed to typical "pepsin agglutinator" reactions, easily inhibited by Fab γ globulin Absorption using Fab(a) and Fab(f) anti D removed "pepsin agglutinator" and the corresponding anti-antibody activity detected by whole anti D A similar pattern was obtained with anti antibody (a, b, f) serum 141 in reactions with Gm(a) and Gm(f) anti D or the Fab fragments hereof The reactions with Gm(b) anti D were rather weak and the results inconclusive

TABLE 3

Titres of Anti Antibody(f) Serum 55 in Saline (A) in Whole γ Globulin (B) and in Fab γ Globulin (C) before and after Various Absorptions

		Test with red cells sensitized by whole Gm(a) anti D	whole Gm(b) anti D	whole Gm(f) anti D	Fab(a) anti D	Fab(b) anti D	Fab(f) anti D
Absorption with red cells							
unsensitized	A	<2	<2	64	16	16	128
	B			64	16	16	128
	C			64	<2	<2	128
sensitized by whole anti D	A	<2	<2	<2	16	8	32
	B				8	8	32
	C				<2	<2	<2
sensitized by Fab anti D	A	<2	<2	<2	<2	<2	<2

For absorptions with sensitized red cells see text
Controls as in Table 1

TABLE 4

Titres of Anti Antibody (a f) Serum 356 in Saline (A) in Whole γ Globulin (B) and in Fab γ Globulin (C) before and after Various Absorptions

		Test with red cells sensitized by whole Gm(a) anti D	whole Gm(b) anti D	whole Gm(f) anti D	Fab(a) anti D	Fab(b) anti D	Fab(f) anti D
Absorption with red cells							
unsensitized	A	32	<2	16	32	<2	32
	B	32		16	32		32
	C	32		16	32		32
sensitized by whole anti D	A	<2	<2	<2	16	<2	16
	B				16		16
	C				<2		<2
sensitized by Fab anti D	A	<2	<2	<2	<2	<2	<2

For absorptions with sensitized red cells see text
Controls as in Table 1

These findings further showed that anti antibody activity in the present sera was detected by Fab anti D

In the agglutination experiments anti antibody reactions were only obtained with the Fab fragment of the anti D used to detect the anti antibody, indicating that their specificity was related to heterogenic determinants of the Fab fragment.

This was sustained by the further results of absorptions using Fab (a), Fab(b) and Fab(f) anti D The anti antibody f 55 was only removed by Fab(f) anti D (Table 5) and absorptions on anti antibody (f) serum 576 gave comparable results

TABLE 5

Titres of Anti Antibody Sera 55 and 356 before and after Absorptions Using Fab Anti D of Different Specificities

	Serum 55 Test with		Serum 356 Test with			
	whole Gm(f) anti D	Fab(f) anti-D	whole Gm(a) anti D	Fab a anti D	whole Gm f anti-D	Fab f anti-D
Absorption with red cells unsensitized	32	32	32	32	16	16
sensitized by						
Fab(a) anti D	16	32	<2	<2	16	16
Fab(b) anti D	32	32	16	16	16	16
Fab(f) anti D	<2	<2	16	16	<2	<2

Each of the detected activities was of anti antibody type Controls as in Table 1

Anti antibody (a f) serum 356 was then absorbed and the activities were tested with whole Gm(a) and Gm(f anti D and with the corresponding Fab fragments These absorptions also demonstrated the strict specificity of anti antibodies for determinants of the Fab fragments Furthermore a crossabsorption was obtained similar to that demonstrated previously (cfr 21 Table 2) by using whole anti D

Test with γM Antibodies

Since γM antibodies give no subagglutinating sensitization of red cells (27) only absorption experiments were performed

Each of the 10 anti antibody sera was absorbed by each of 5 batches of sheep red cells sensitized by 5 individual γM antibodies respectively Activity in a given serum was tested using Gm(a) Gm(b) and Gm(f) anti D No anti antibody was absorbed by any of the red cells sensitized by γM-antibodies The titres of the absorbed sera and controls never differed more than one step After the absorption procedures each of the activities was still of anti antibody nature Immune complexes containing γM antibodies thus did not react with anti-antibodies

Test with Aggregated γ Globulin

Heat aggregated whole γ globulin (6 mg/ml), pepsin digested γ globulin (4 mg/ml) and papain digested γ globulin (6 mg ml) were used in inhibition experiments with the 10 anti antibody sera A constant amount of aggregated material was added to twofold dilutions of anti antibody sera The agglutination was compared to that in saline Aggregated whole γ globulin or the papain digested fragments (Fab and Fc) did not inhibit anti antibody activity in any of the sera while anti Gm(a) activity present in 2 sera (355 and 700) completely disappeared Furthermore aggregates of pepsin digested γ globulin (Fab fragments) did not inhibit the anti antibodies but showed complete inhibition of pepsin agglutinators tested as controls In further controls no inhibition of anti antibody activity was obtained with the corresponding unaggregated whole γ globulin or its digested products

Experiments with Blocking of the Reacting Sites

Red cells were sensitized by the reference whole Gm(a) Gm(b) or Gm(f) anti D and each type of sensitized red cells divided in 4 batches Five volumes of anti antibody sera 55 or 141 diluted 1 2 or the same amounts of saline (for control) were added to one volume of each type of packed sensitized red cells The mixtures were kept at room temperature for 2 hours After removal of the supernatant fluids and thorough washing of the red cells the same amounts of anti antibody serum as before were added After repeated washing each batch of red cells was divided One portion was used for the absorption of an equal volume of anti antibody serum diluted 1 2 and after this control absorption the anti antibody activity was maintained The second portion of sensitized red cells however easily removed an anti Gm factor corresponding to the Gm specificity of the anti D on the red cells Gm(f) anti D sensitized red cells saturated with anti antibody 55 or 141 absorbed anti Gm(f) Similarly Gm(a) anti D sensitized red cells treated with the same anti antibodies removed anti Gm(a) Red cells sensitized by Gm(b) anti D gave comparable results

In controls saline treated sensitized red cells easily removed the corresponding anti antibodies and anti Gm factors Furthermore unsensitized red cells treated with the respective anti antibody sera in the same way to the sensitized red cells did not remove the anti Gm activity

Similarly anti antibodies were absorbed by sensitized red cells which had been saturated with anti Gm factors The findings indicated that the anti antibodies and anti Gm factors involved different sites of the γ globulin molecules The experiment was however not repeated since large quantities of important sera were necessary

DISCUSSION

Harboe et al (8) recently demonstrated "pepsin agglutinators" in all their 5 anti-antibody sera In the present study 9 out of 10 such sera contained "pepsin agglutinators" The only anti antibody serum which lacked "pepsin agglutinator" originated from a young healthy blood donor and the anti-antibody titre was relatively low

The anti antibodies and "pepsin agglutinators" were separable by absorptions and agglutination inhibition tests There may, however, be a close relationship between the 2 types of anti-γ-globulin factors since "pepsin agglutinators" are so common in anti-antibody sera Furthermore, a "pepsin agglutinator" was always related to the anti-D used to detect an anti-antibody in the same serum

"Pepsin-agglutinators" have been found in different γ-globulin classes and are often γG globulins (24) However, in sera with rheumatoid factors there was a predilection to find "pepsin agglutinators" in the γM-globulin class (26) In the present selected material all the "pepsin agglutinators" were γM globulins as judged from loss of activity after treatment by mercaptoethanol Since the anti-antibodies were themselves γM-globulins, this further showed the relationship between the 2 types of anti γ-globulin factors

Individual specificity of "pepsin agglutinators" in sera without anti-antibody activity was recently demonstrated (22) "Pepsin-agglutinators" reacting only with one type of Fab anti D (monospecific) were frequent The present study showed individual specificity of "pepsin agglutinators" in anti antibody sera No anti-antibody serum contained only one monospecific "pepsin agglutinator", but 2 "pepsin-agglutinator"(a, f) sera were found The "pepsin agglutinators" in anti-antibody sera were thus more polyspecific than in sera without anti-antibody activity The existence of "pepsin agglutinators"(a f) may be explained in accordance with previous findings indicating that group specific determinants are involved in their reactions (22)

All the anti antibodies investigated reacted with Fab anti D in immune complexes but not with Fab γ globulin in solution *Fudenberg et al* (4) showed that also rabbit anti-antibodies reacted with Fab antibodies of isologous origin However, *Harboe et al* (8) found a human anti antibody reacting with sites on the Fc fragments, and *Natvig* (22) found another type detected exclusively by Fab antibodies Thus different classes of anti antibodies exist

The anti antibody specificity was related to the Gm type of the anti D used to detect the anti antibody (20) The present findings demonstrated a strict relation of anti antibody specificity to one whole anti D and its corresponding Fab fragments This indicated that anti-antibodies were directed against heterogenic determinants on the Fab fragment, corresponding to those of the whole molecule Since the Gm types, used as markers in these studies (see 18, 20), belong to the

heavy chains of γG-globulin (5, 12) the anti-antibodies probably indicate heterogenic determinants of the Fd fragment The finding of inhibition of similar factors in rabbits with heavy but not with light chains (29) was in accordance with this view.

The present and previous findings (see 22) of heterogeneity of the Fd fragment related to determinants specific only for γG globulin, indicated that the Fd fragment differed in γG- and other immunoglobulins A corresponding finding was recently reported (7) Furthermore, Gm(f) which is probably very close to determinants involved in anti-antibody reaction, belong to a part of the heavy chain which is not shared in common by γG-, γA- and γM-globulins (5) Although the Fd piece of the γG- and γM-globulins may share some antigenic determinants (23), various other findings indicate that the structure of antigen binding sites is different in γM- and γG globulins (see 7) That anti-antibodies did not react with γM-antibodies (see also 8, 17) was thus compatible with involvement of the Fd fragment in anti antibody reaction

Further information can be obtained from the inhibition reactions When Fab γ globulin was added to anti-antibody sera containing "pepsin-agglutinators", soluble Fab γG globulin—"pepsin-agglutinator" complexes were probably formed Anti-antibody molecules were, however, not absorbed by these complexes since anti-antibody reaction with Fab anti-D was not inhibited by Fab γG-globulin The reaction was at least as high as with corresponding whole anti-D (see Tables 1 and 2) Similarly when whole γ-globulin was added to anti antibody sera containing anti Gm factors, soluble whole γG-globulin anti Gm complexes were formed However, the anti-antibody titres of the respective sera were not reduced These immune complexes contained human γG-globulin as antigen and human γM-globulin as antibody The observations thus further indicated that anti-antibodies did not react with immune complexes containing isologous γM-globulin as antibody They also confirmed the findings of Harboe et al (8) that anti-antibodies did not react with human γG-globulin acting as antigen in an immune complex Tests should, however, be performed, using human γG globulin and γG-anti-γ-globulin factors

Two findings further excluded the possibility that the anti antibody reaction with Fab anti-D was caused by immune complexes including "pepsin-agglutinators" 1 One serum lacked "pepsin-agglutinator" but still showed strong anti antibody reaction with red cells sensitized by Fab anti D 2 Anti-antibodies showed specificity for one Fab anti D although "pepsin-agglutinators" in the same sera reacted with other Fab anti-D antibodies

During denaturation changes occur in the γ-globulin molecule (1 2) probably most extensively in the Fc part, causing rupture of inter chain disulphide bonds with intermolecular bridging and exposure of new chemical groupings (9) In rabbit anti-antibodies Fudenberg et al (4)

demonstrated some inhibition of anti antibody activity with aggregate isologous Fab γ-globulins No corresponding inhibition of human ant antibodies was obtained by Harboe et al (8) or in the present study Conventional heat aggregation is thus probably not able to reproduc the changes which occur in the antibody molecules during combinatio with antigen (11, 25) Such specific changes seem to be necessary fo anti-antibody reaction (8, 16, 17)

The relation of anti-antibody specificity to determinants of the F fragment is a new indication that the antigen binding site is locate to that part of the molecule (see 3) The specific changes necessar for anti-antibody reaction will also probably occur in the Fd fragment This is in accordance with a recent suggestion by *Henney et al* (10 that "new antigenic determinants, revealed by specific antigen anti body combination, are located on the Fd piece of the antibod molecule"

No blocking of the Gm site was obtained with anti-antibodies, an of site for anti-antibody reaction with anti-Gm factors However, i the experiment an interaction between 2 types of anti-γ-globulin factor causing anti-Gm—anti antibody complexes on the red cell surface coul not be excluded This is not likely since the anti-Gm factors and anti antibodies used were γM-globulins while they showed antibody spe cificity only for γG globulin The findings thus indicated that the site (determinants) involved in the anti antibody and anti Gm reaction were separate Close connection has been established between anti antibody specificity and certain Gm types and it is thus possible tha the antigen binding site, as the Gm types, shows genetically determine heterogeneity

Both physicochemical and serological properties of the anti anti bodies and "pepsin agglutinators" have previously indicated the antibody characters (14 17, 24, 26) This is further supported b findings of their individual specificity, one of the main characteristic of antibodies (28) More extensive studies on the specificities of anti antibodies *and "pepsin agglutinators"* are currently in progress

SUMMARY

1 "Pepsin-agglutinators" were demonstrated in 9 out of 10 anti antibody sera The "pepsin agglutinators" usually showed a broade specificity than anti antibodies in the same sera, and were generall more polyspecific than those in sera without anti-antibodies

2 Anti antibodies reacted both with whole and pepsin-digested (Fab fragment) incomplete anti D acting as antibody in immune complexes They showed a strict specificity for determinants of some anti-D anti bodies and their corresponding Fab fragments Anti antibodies thu indicated heterogeneity of the Fab fragment of γG-globulin, probabl the Fd piece

Acta path et microbiol scandinav 66 396–400 1966

The Institute of Virology Uppsala University, Uppsala and the National Veterinary Institute Stockholm Sweden

EFFECT OF THYMECTOMY ON TUMOUR PRODUCTION BY POLYOMA VIRUS IN RABBITS

By

HANS DIDERHOLM TIMO ESTOLA and TORI WESSLÉN

Received 10 xii 65

Polyoma virus is capable of inducing malignant tumours in a variety of species Mouse (*Gross* 1953, *Stewart et al* 1957, *Mirand et al* 1958) rat (*Eddy et al* 1959b, *Kirsten et al* 1962), hamster (*Defendi* 1960, *McCulloch et al* 1961, *Barski et al* 1962), guinea-pig (*Eddy et al* 1960) ferret (*Harris et al* 1961), and *Mastomys* (*Rabson et al* 1960) When polyoma virus is injected into rabbits, benign tumours develop at the site of injection (*Eddy et al* 1959a) These tumours regress within a few weeks

Recently it was shown that thymectomy in the neonatal period influenced the tumour induction by polyoma virus in rats (*Vandeputte et al* 1963) and mice (*Malmgren et al* 1964, *Miller et al* 1964, *Law* 1965) In thymectomized animals the incidence of tumours was higher than in sham-operated controls Thymectomized rats also showed prolongation of the period during which the animals were susceptible to the oncogenic effect of the virus

The present work deals with the effect of thymectomy on the tumour production by polyoma virus in rabbits It is shown that the virus has an increased oncogenic activity in the thymectomized animals

MATERIAL AND METHODS

Animals Albino rabbits not older than 24 hours were used Half the an[illegible]mals were thymectomized and the remainder were sham operated Animals of the [illegible]ame litter were ranked according to their weight and so placed that both the experim[illegible]ntal and the control group contained all types from the heaviest to the lightest

Operation technique The animals were anaesthetized with ether an[illegible] [illegible]hymectomized through a median incision of the chest wall The thymus was [illegible] by surgical dissection and the incision was closed with clips Sham [illegible] was carried out in a similar way but the thymus was left *in situ*

Virus T polyoma virus was used (The virus was kindly supplied [illegible] Pr[illegible] M Stoker *Glasgow*) *It had had several passages in our laboratory in cultu*[illegible] [illegible] *mouse* embryo cells

Assay of virus The haemagglutination test was used t[illegible] estimate [illegible] [illegible]oncentration To serial twofold dilutions of 0.5 ml of virus 0.5 ml of an [illegible] per cent

This investigation was supported by grants from the Swedish Canc[illegible] *Society*

suspension of guinea pig red blood cells was added The tubes were kept at 4° C overnight The virus titre was the highest dilution giving complete agglutination

Injection with virus The animals were given polyoma virus within 2–4 hours after the operation They were injected subcutaneously in the nuchal area with 1 ml per 100 g body weight of a virus suspension having a haemagglutination titre of 1/2048

Examination of the tumours The animals were examined twice weekly The tumour was measured and two diameters noted, the largest one and also the largest perpendicular to it The mean of these two diameters was calculated and designated the mean tumour diameter (MTD)

Assay of haemagglutination inhibiting antibodies To serial twofold dilutions of 0 25 ml of heat inactivated serum, 0 25 ml of a virus suspension containing 4 haem agglutinating units was added The mixtures were kept at 37° for 60 minutes and after the addition of blood cells at 4° C overnight before readings were made The inhibition titre was the highest dilution completely inhibiting agglutination

RESULTS

As seen in Table 1, all eleven thymectomized rabbits developed tumours whereas in the control group these were noted in eight out of ten animals There was no difference in the latent period between the groups, the mean period in the thymectomized group being 22 6 ± 1 1 days and in the control group 23 4 ± 0 8 days (Table 1)

TABLE 1

Incidence and Behaviour of Tumours in Thymectomized and Sham Operated Rabbits Inoculated with Polyoma Virus

Operation	Number of animals		Tumours		
	inoculated	with tumours	Time of appearance*	Time of disappearance†	Size§ (mm)
Thymectomy	11	11	22 6 ± 1 1	107 2 ± 23 9	34 3 ± 4 8
Sham	10	8	23 4 ± 0 8	28 5 ± 3 2	17 5 ± 2 4

* Mean time in days after inoculation of polyoma virus
† Mean time in days after appearance of tumours
§ Mean of maximal MTD (mean tumour diameter) of each individual tumour

In Fig 1 the means of MTD (mean tumour diameters) at different times after inoculation are plotted The highest value in the thymectomized group was 27 4 millimeter and this was reached by the 50th day after infection In the sham-operated group the highest value, 13 5 millimeter, was noted on the 40th day after infection

The mean of the maximal MTD of each individual tumour was also calculated (Table 1) In the thymectomized group this was 34 3 ± 4 8 millimeter and in the sham-operated controls 17 5 ± 2 4 millimeter This difference is statistically significant $(0 01 > P > 0 001)$

As also seen in Fig 1 the time for the disappearance of the tumours was considerably longer in the thymectomized group than in the sham-operated controls In the former all tumours had regressed completely within 244 days after the infection, whereas in the sham-operated group the corresponding period was 71 days

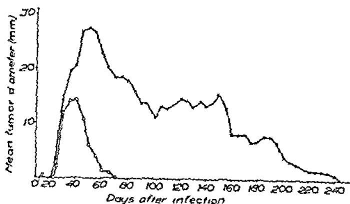


Fig 1

Growth of tumours in thymectomized (X) and sham operated (O) rabbits inoculated with polyoma virus

The mean of the times during which the animals showed tumours was in the thymectomized group 107 2 ± 23 0 days and 28 5 ± 3 2 days in the sham-operated group (Table 1) This difference is statistically significant (0 01 > P > 0 001)

There was no difference in haemagglutination inhibiting antibody titres between the thymectomized and sham-operated rabbits The titres varied between 1/320 and 1/5120

The thymectomy did not influence the body weight of the animals during the course of the investigation

DISCUSSION

The data presented above clearly show that the thymectomized rabbits developed larger tumours than did sham-operated controls after the inoculation of polyoma virus In addition, the tumours of the thymectomized animals were palpable for a considerably longer period than those of the controls

As mentioned previously other studies have been made on the influence of neonatal thymectomy on tumour formation by polyoma virus Both in rats and mice the incidence of tumours was higher in the thymectomized than in the control group (*Vandeputte et al* 1963 *Malmgren et al* 1964, *Miller et al* 1964, *Law* 1965) In neither of these species nor in the rabbits did thymectomy influence the haemagglutination inhibiting antibody titres This fact supports the assumption that the effect of thymectomy on the tumour development was due to an impairment of the immunologic response against foreign cellular antigens present in the tumours (*Sjogren et al* 1961 *Habel* 1962) rather than to a depression of the humoral immunity

It has been shown that the effect of neonatal thymectomy in rabbits is variable and often weak compared with its effect in certain strains of mice, rats and hamsters (*Good et al* 1962, *Archer et al* 1962) The

reason for this may be that the appendix of the rabbit has a similar function to the bursa of Fabricius in the chicken (*Archer et al* 1963) It may be asked whether polyoma virus in rabbits neonatally thymectomized and appendectomized is capable of producing tumours which will continue to grow and become malignant Experiments are under way to examine this possibility At present it seems, however, that the tumours will regress in the thymectomized and appendectomized rabbits also

SUMMARY

The tumour formation by polyoma virus was studied in rabbits thymectomized and inoculated with the virus within 24 hours after birth

It was shown that the thymectomized animals developed larger tumours than did the sham operated controls In both groups the tumours regressed completely, in the thymectomized group after a considerably longer period than in the control group

REFERENCES

1 *Archer O K Pierce J C Papermaster B W & Good R A* Reduced Antibody Response in Thymectomized Rabbits Nature *195* 191–193 1962

2 *Archer O K Sutherland D E R & Good R A* Appendix of the Rabbit A chicken? Nature *200* 337 339 1963

3 *itsky T & Cralier M* Neoplasms and in Adult Hamsters with Polyoma Virus

4 Polyoma in Adult Hamsters Nature *188* 508 1960

5 *Eddy B E Borman G S Kirschstein R L & Touchette R H* Neoplasms in Guinea Pigs Infected with SE Polyoma Virus J Inf Dis *107* 361–368 1960

6 *Eddy B E Stewart S E Kirschstein R L & Young R D* Induction of Subcutaneous Nodules in Rabbits with SE Polyoma Virus Nature *183* 766 767 1959 a

7 *Eddy B E Stewart S E Stanton M F & Marcotte J M* Induction of Tumors in Rats by Tissue Culture Preparation of SE Polyoma Virus J Natl Cancer Inst 22 161–171 1959 b

8 *Good R A Dalmasso A P Martine C Archer O K Pierce J C & Papermaster B W* The Role of the Thymus in Development of Immunologic Capacity in Rabbits and Mice J Exptl Med *116* 773 795 1962

9 *Gross L* A Filterable Agent Recovered from AK Leukemic Extracts Causing Salivary Gland Carcinomas in C3H Mice Proc Soc Exptl Biol Med *83* 414 421 1953

10 *Habel K* Immunological Determinants of Polyoma Virus Oncogenesis J Exptl Med *115* 181–193 1962

11 *Harris R J C Chesterman F C & Negroni G* Induction of Tumors in New born Ferrets with Mill Hill Polyoma Virus Lancet *1* 788 791 1961

12 *Kirsten W H Andersson D G Platz C F & Crowell Jr E B* Observations on the Morphology and Frequency of Polyoma Tumors in Rats Cancer Res 22 284–491 1962

13 *Law L W* *Thymus* Role in Resistance to Polyoma Virus Oncogenesis Science *147* 164–165 1965

14 *Malmgren R A Rabson A S & Carney P G* Immunity and Viral Carcinogenesis Effect of Thymectomy on Polyoma Virus Carcinogenesis in Mice J Natl Cancer Inst 33 101 104 1964

15 *McCulloch E A Siminovitch L Ham A W Axelrad A A & Howatson A F* Carcinogenesis *in vitro* by Polyoma Virus Canadian Cancer Conference 4 253 270 1961

16 *Miller J F A P Ting R C & Law L W* Influence of Thymectomy on Tumor Induction by Polyoma Virus in C57BL Mice Proc Soc. Exptl Biol Med *116* 323 327 1964
17 *Mirand E., Mount D T., Moore G E Grace J T & Sokal J F* Induction of Tumors by Virus Like Agents Released by Tissue Culture Proc Soc Exptl. Biol Med. *99* 1 5 1958
18 *Rabson A S., Branigan W J & Legallais F Y* Production of Tumours in *Rattus (Mastomys) natalensis* by Polyoma Virus Nature *187* 423–425 1960
19 *Sjogren H O Hellstrom I & Klein G* Resistance of Polyoma Virus immunized Mice Against Transplantation of Established Polyoma Tumors Exptl Cell Res *23* 204–208 1961
20 *Stewart S E., Eddy B E Gochenour A M., Borgese N G & Grubbe G F* The Induction of Neoplasms with a Substance Released from Mouse Tumors by Tissue Culture Virology *3* 380–400 1957
21 *Vandeputte M Denys Jr P Leyton R & De Somer P* The Oncogenic Activity of the Polyoma Virus in Thymectomized Rats Life Sciences *7* 475–478 1963

Acta path et microbiol scandinav 66 401 408 1966

Departments of Pathology Central Hospital Borås and General Hospital Malmö
Sweden

GENERALIZED HERPES SIMPLEX IN NEWBORNS

By

S Szogi and Th Berge

Received 31 viii 65

In 1935 *Hass* described a previously unknown disease in a newborn child The disease had a peculiar pathological picture characterized by miliary necrotic foci in the liver and adrenals Owing to the occurrence of intranuclear eosinophilic inclusion bodies in the areas of the necrotic foci he thought that these lesions were caused by herpes simplex virus He also found the gross as well as the microscopic picture to resemble that seen in rabbits experimentally infected with the virus (*Goodpasture et al* 1923)

In 1952 *Zuelzer & Stulberg* published 8 cases with identical visceral lesions In one of these cases they demonstrated the presence of herpes simplex virus

The first case known in Scandinavia is that reported by *Ericsson et al* (1958) *Langvad & Voigt* (1963) traced 53 cases in the literature up to 1963 including 17 verified virologically

CASE REPORTS

Case 1

the liver was enlarged and the child died despite treatment with gammaglobulin cortisone and penicillin

Necropsy

Gross findings A full term child with no malformations No cutaneous vesicles Widespread ulcerations were seen in the lower half of the oesophagus The lungs showed purple foci the size of rice seeds Scattered greyish white necrotic foci the size of pinheads were seen on the surface and on the cut surface of the liver (wt 150 g) The foci were surrounded by haemorrhagic haloes The adrenals appeared normal Well defined greyish white patches were seen in the red marrow of the vertebrae The cerebellar cortex and the soft membranes over the cerebellar hemispheres showed numerous punctate haemorrhages

Microscopic findings There were moderate diffuse deposits of leucocytes and

a few small rounded areas of gliosis Vascular changes with verrucose fibrin deposits were found also in the pons

The vertebral bodies were sites of small, well defined necrotic foci involving the cortex and the bone marrow but showed no signs of inflammatory reaction

Minute necrotic foci were seen in the subcapsular layer of the adrenal cortex

The liver contained numerous, crowded and sometimes coalescent, necrotic foci 2–3 mm in diameter, with haemorrhagic margins The periportal connective tissue was loose and contained some inflammatory cells Immediately adjacent to the necrotic foci the liver cells contained intranuclear eosinophilic inclusion bodies of Cowdry's type A Scattered multinuclear liver cells were also seen.

The ulcerations in the oesophagus were coated with fibrin with intermingled inflammatory cells Proliferating fibroblasts and vessels with swollen endothelium were observed beneath the floor of the ulcerations

In the lungs were groups of alveoli containing red blood cells Peribronchial clusters of inflammatory cells were also observed

Virological examination (performed at the Virological Laboratory, Sahlgren's Hospital Gothenburg) Herpes simplex virus was isolated from cell cultures of necropsy specimens of the liver, kidney, liver, lung and intestines A substance producing herpes simplex like lesions was isolated from fertilized hen's egg (chorio-allantoic membrane) Repeated attempts to isolate the virus from the faeces failed

Serological examination of the mother revealed lack of neutralizing antibodies to herpes simplex virus

Inquiry among the staff of the department of obstetrics revealed that one of the nurses had for a long time had obstinate, recurrent vesicles on the lips (herpes labialis)

Case 2

The mother was a Para I She had had poliomyelitis at 7 years of age Appendectomy at 13 Treated for gonorrhoea during pregnancy Spontaneous labour and rupture of the membranes 11 weeks before calculated term Because of secondary weakness of labour she was delivered 2 days later with the aid of a vacuum extractor and low forceps The child a premature baby boy (wt 2,200 g length 47 cm) was asphyctic immediately after birth but soon recovered On the third day he was jaundiced and the serum bilirubin gradually increased (max 34 mg/100 ml) Hb 16 4 mg/100 ml Reticulocytes 1.2 *per cent* Coomb s test was negative No serological evidence of Rh or ABO immunization The child was transferred to the department of paediatrics where the child s blood was exchanged The jaundice was interpreted as icterus neonatorum which had been accentuated by premature birth After the transfusion the serum bilirubin successively decreased Body temperature was normal except on the tenth and eleventh days when it fell to 34 5° C The child was tube fed with the mother's milk From about the ninth day the child was increasingly listless The respiration was weak and the heart beat slow but no neurological signs were manifest On the tenth day the bilirubin had fallen to 12 1 mg 100 ml Vomiting supervened and the child died on the eleventh day

Necropsy

Gross findings Yellow discoloration of the skin and mucosae Internal organs icteric No cutaneous or mucosal vesicles No malformations Bile ducts of normal appearance The liver weighed 135 g Its surface as well as its cut surface showed numerous scattered rounded greyish white lesions 2 3 mm in diameter and surrounded by haemorrhagic haloes

The adrenal cortex exhibited the same type of lesions as the liver

Microscopic findings The lesions in the liver were focal but not localized to single acini The reticular network and the vascular pattern were obliterated An accumulation of blood was seen in dilated sinusoids around the foci Otherwise the necrotic foci were without any demarcating inflammatory reaction Around the foci were a number of multinuclear hepatic cells indicating a tendency to regeneration In the immediate neighbourhood of the lesions were numerous cells with intranuclear eosinophilic inclusion bodies surrounded by a light halo The chromatin was condensed along the nuclear membranes

The lesions in the adrenals which were of the same histological appearance were strictly confined to the cortex

A few m ddle s zed branches of the pulmonary artery showed verrucose deposits of f brin and necrosis of the endothel al cells

No focal changes could be found in the brain.

The mother declared that she had never had herpes s mplex Analys s of the mother s serum 2 months after partur t on revealed lack of neutral z ng antibod es to herpes s mplex virus

DISCUSSION

Generalized herpes simplex like Kaposi s varicelliform eruption herpes encephalitis vulvo vaginitis and gingivostomatitis belongs to the group of rare primary forms of herpes liable to occur in persons lacking neutralizing antibodies to the herpes simplex virus All of these primary infections are serious as compared with the common secondary herpes simplex (*e g* herpes labialis) Like other infections in the newborn herpes simplex does not produce any characteristic clinical symptoms but rather a picture which varies according to the organ or organs affected The disease makes its appearance in the first or second week of life and as a rule usually terminates in death within a few days The few who survive have severe permanent nervous sequelae (*Florman & Mindlin* 1952 *Epstein & Crouch* 1954) The symptoms described in neonatal herpes simplex *e g* fever hypothermia dyspnoea cyanosis hepatomegaly splenomegaly jaundice and convulsions are also prone to accompany other infections such as Coxsackie B virus infection cytomegalia infantum toxoplasmosis or bacterial sepsis The diagnosis can be made when vesicles occur on the skin or mucosae The virus can also be isolated *in vivo* from faeces Attempts have also been made to confirm the diagnosis by histological examination of liver biopsy specimens

The pathological picture is pathognomonic and in many cases on record the diagnosis was not made until after death The necrotic foci are most prominent in the liver and the adrenals but are also demonstrable in other organs such as the oesophagus spleen lungs brain kidneys and bone marrow The lesions are rounded and 2 3 mm in diameter (Fig 1) Microscopically they show the picture of coagulation necroses saturated with blood In the necrotic areas the mesenchymal structures (endothelial cells vascular walls and reticular network) are often destroyed though the reticular network is sometimes preserved *Langt ad & Voigt* (1963) The foci are not encircled by inflammatory cells (*Fig 2*)

In the liver the necroses are focal and not confined to single acini A single necrotic focus often involves parts of several acini Cells with eosinophilic intranuclear inclusion bodies of Cowdry s type A occur around the necroses The inclusion bodies are surrounded by a light halo bordered by a compact chromatin layer pressed against the nuclear membrane Inclusion bodies are most readily detected in the liver because of the vesicular nature of the hepatic nuclei (Figs 3 and 4) Beside these compact eosinophilic inclusion bodies a smaller number

a few small rounded areas of gliosis Vascular changes with verrucose fibrin deposits were found also in the pons

The vertebral bodies were sites of small well defined necrotic foci involving the cortex and the bone marrow but showed no signs of inflammatory reaction

Minute necrotic foci were seen in the subcapsular layer of the adrenal cortex

The liver contained numerous crowded and sometimes coalescent necrotic foci 2–3 mm ir [illegible]
was loose [illegible]
necrotic f [illegible]
Cowdry s [illegible]

The ulcerations in the oesophagus were coated with fibrin with intermingled inflammatory cells Proliferating fibroblasts and vessels with swollen endothelium were observed beneath the floor of the ulcerations

In the lungs were groups of alveoli containing red blood cells Peribronchial clusters of inflammatory cells were also observed

Virological examination (performed at the Virological Laboratory, Sahlgren s Hospital Gothenburg) Herpes simplex virus was isolated from cell cultures of necropsy specimens of the liver kidney liver lung and intestines A substance producing herpes simplex like lesions was isolated from fertilized hen s egg (chorio-allantoic membrane) Repeated attempts to isolate the virus from the faeces failed

Serological examination of the mother revealed lack of neutralizing antibodies to herpes simplex virus

Inquiry among the staff of the department of obstetrics revealed that one of the nurses had for a long time had obstinate recurrent vesicles on the lips (herpes labialis)

Case 2

The mother was a Para I She had had poliomyelitis at 7 years of age Appendectomy at 13 Treated for gonorrhoea during pregnancy Spontaneous labour and rupture of the membranes 11 weeks before calculated term Because of secondary weakness of labour she was delivered 2 days later with the aid of a vacuum extractor and low forceps The child a premature baby boy (wt 2 200 g length 47 cm) was asphyctic immediately after birth but soon recovered On the third day he was jaundiced and the serum bilirubin gradually increased (max 34 mg/100 ml) *Hb* 16 4 mg/100 ml Reticulocytes 1 2 per cent Coomb s test was negative No serological evidence of Rh or ABO immunization The child was transferred to the department of paediatrics, where the child s blood was exchanged The jaundice was interpreted as icterus neonatorum which had been accentuated by premature birth After the transfusion the serum bilirubin successively decreased Body temperature was normal except on the tenth and eleventh days when it fell to 34 5° C The child was tube fed with the mother s milk. From about the ninth day the child was increasingly listless The respiration was weak and the heart beat slow but no neurological signs were manifest On the tenth day the bilirubin had fallen to 12 1 mg/100 ml Vomiting supervened and the child died on the eleventh day

Necropsy

Gross findings Yellow discoloration of the skin and mucosae Internal organs icteric No cutaneous or mucosal vesicles No malformations Bile ducts of normal appearance The liver weighed 135 g Its surface as well as its cut surface showed numerous scattered rounded greyish white lesions 2 3 mm in diameter and surrounded by haemorrhagic haloes

The adrenal cortex exhibited the same type of lesions as the liver

Microscopic findings The lesions in the liver were focal but not localized to single acini The reticular network and the vascular pattern were obliterated An accumulation of blood was seen in dilated sinusoids around the foci Otherwise the necrotic foci were without any demarcating inflammatory reaction Around the foci were a number of multinuclear hepatic cells indicating a tendency to regeneration In the immediate neighbourhood of the lesions were numerous cells with intranuclear eosinophilic inclusion bodies surrounded by a light halo The chromatin was condensed along the nuclear membranes

The lesions in the adrenals which were of the same histological appearance were strictly confined to the cortex





Fig 4 *Fig 3*

Figs 3 4

Inclusion bodies in nuclei of hepatic cells adjacent to necrotic foci (× 380 × 972) Haematoxylin eosin

of inclusions are observed such as compact basophilic aggregates and eosinophilic intranuclear granules with a light zone round each granule The last mentioned variants are regarded as developing or degenerating inclusion bodies

The necroses in the adrenals are always situated in the cortex

Changes in the oesophagues appear in the form of superficial ulcerations with the above mentioned type of inclusion bodies along the margin and in the floor of the lesions (*Zuelzer & Stulberg* 1952 *Ericsson et al* 1958) In the floors of the lesions the inclusion bodies are most often situated in endothelial cells Visceral lesions are sometimes accompanied by encephalitis If so the picture is dominated by affection of the cerebral capillaries with necrosis of the endothelial cells and fibrin thrombi in their lumina (Fig 5) Haemorrhages or necrotic foci are seen adjacent to these vascular changes Inclusion bodies sometimes also occur both in the glia and the ganglion cells Similar changes are seen in the pulmonary vessels (Fig 6)

In the newborn the actual infection with herpes probably occurs after parturition as the incubation period of primary herpes simplex is 3 days and the initial clinical symptoms often appear on the fourth day of life The possibility of intrauterine infection has however been pointed out by *France & Wilmers* (1953) and by *McDougal et al*

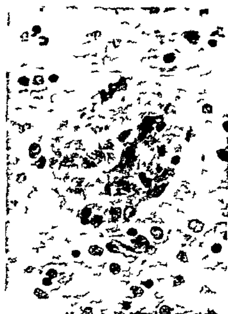




Fig 5 Fig 6

Figs 5–6

Necrosis of endothelial cells and fibrinoid deposits in cerebral vessels and branch of pulmonary artery (× 243 × 11) Haematoxylin eosin

(1945) *Biegeleisen et al* (1962) demonstrated the transplacental passage of herpes virus in rabbits The source of infection is to be looked for in the child s entourage particularly among the personnel in the department of obstetrics *Bird et al* (1959 1963) traced contact with persons infected with herpes in 5 of their 6 cases

The skin mucous membranes oesophagus umbilicus and airpassages have been considered as possible portals of entry of the virus The fairly regular involvement of the liver argues for infection via the umbilical cord

The titre of neutralizing and complement fixing antibodies to herpes simplex virus in the mother s blood is the same as that in the foetal child s blood The immunity is transferred via the placenta to the child gradually to disappear between the third and seventh month During this time most children are immune to herpes *Hol el et al* (1953)

In densely populated areas 86 per cent of the adult population have antibodies to herpes simplex

Mothers of newborn children with generalized herpes usually have no such antibodies *Bird et al* (1959) and *McCallum* (1959) have however published cases in which the mothers had antibodies in high titres In the first publications the infants were often premature but later the infection has been reported to occur in children of normal bodyweight Neither prematurity nor lack of antibodies can therefore

be regarded as an obligatory condition for the development of herpes simplex infection in newborn children

The visceral necroses described above occur not only in the neonatal period but also in various other forms of primary herpes in older children (*Epstein & Crouch* 1954 *Zuelzer & Stulberg* 1952)

The gross and microscopic findings usually allow differentiation of the condition from other known viral infections in newborn children Coxsackie B virus always causes myocarditis and is never accompanied by the appearence of inclusion bodies *Szogi & Bergstrom* (1962)

Cytomegalia infantum can fairly easily be recognized by its characteristic features and by the occurrence of intranuclear as well as intracytoplasmic inclusions It may, however, sometimes be difficult to distinguish between herpes and generalized varicellae The gross picture of generalized herpes, also called hepatoadrenal necrosis, the liver and *the adrenals being the organs most commonly affected resembles* listeriosis in some respects, but microscopic differentiation offers no difficulties This also applies to toxoplasmosis

No effective treatment is available Treatment with immunoglobulins and with corticosteroids has been suggested to counteract the effect of adrenal damage *Bird et al* (1963)

SUMMARY

Two cases of generalized herpes simplex with identical gross and macroscopic lesions are described

In the first case the diagnosis was confirmed by isolation of herpes simplex virus *The child had probably been infected by an attending* nurse in whom subsequent inquiry revealed that she had obstinate recurrent vesicles This shows how important it is to protect the new born children from contact with persons presenting evidence of herpes simplex infection

The second case showed that the condition erroneously may be interpreted as icterus neonatorum

REFERENCES

Biegeleisen J Z Scott L V & Joel W Further evidence of fetal infection with herpes simplex virus Am J Clin Path *37* 289 293 1962

Bird T & Gardner P S Disseminated herpes simplex in the newborn Brit med J *58* 993 996 1959

Bird T Ennis J E Wort A J & Gardner P S Disseminated herpes simplex in newborns J Clin Path *16* 423–431 1963

Cowdry E V The problem of intranuclear inclusions in virus diseases Arch Path *18* 527 542 1934

Epstein H C & Crouch W L Herpes simplex of the newborn infant Pediatrics *13* 553–555 1954

Friesson H Itemark B I Johnsson T & Zetterström R Generalized Herpes Simplex Infection Associated with Staphylococcal Septicemia in a Newborn Infant Acta Paediat (Upps) 47 666–674 1958

Florman A L & Mindlin R I Generalized Herpes simplex in an 11 day-old Premature Infant A M A Amer J Dis Child 82 481–485 1952

France N E & Wilmers M J Herpes simplex Hepatitis and Encephalitis in New born Twins Lancet 264 1181 1183 1953

Goodpasture E W & Teague O Experimental Production of herpetic Lesions in Organs and Tissues of Rabbit J Med Res 44 121 138 1923

Hass G M Hepato Adrenal Necrosis with Intranuclear Inclusion Bodies Amer J Path *11* 127 149 1935

Holzel A Feldman G V Tobin J O H & Harper J Herpes Simplex A Study of Complement Fixing Antibodies at Different Ages Acta Paediat (Upps) 42 206–214 1953

Langvad A & Voigt J Herpes generalisata infantum A review and a report of a new case Danish Med Bull *10* 153 158 1963

McCallum F O Generalized herpes simplex in neonatal period Acta virol 3 suppl., 17 21 1959

McDougal R Beamer P R & Hellerstein S Fatal herpes simplex hepatitis in a newborn Amer J Clin Path 24 1250 1258 1954

Szögi S & Bergstrom A L Akut aseptisk meningo encephalit med sannolik Coxsackie etiologi hos nyfodd Nordisk med *68* 1451 1962

Zuelzer W W & Stulberg C S Herpes Simplex as the Cause of Fulminating Visceral Disease and Hepatitis in Infancy A M A Amer J Dis Child *83* 421–439 1952

Acta path et microbiol scandinav 66 409 425 1966

Institute of General Zoology, University of Copenhagen Denmark

HISTOCHEMICAL AND ELECTRONMICROSCOPICAL ASPECTS OF HUMAN BUCCAL MUCOSA

By

Fritz von Bülow

Received 1 ix 65

A comprehensive histochemical study of the non keratinizing, human oral mucosa has never been made The literature contains reports on one or a few histochemical aspects (*Zander* 1941, *Cabrini & Carranza* 1951, *Wislocki, Fawcett & Dempsey* 1951, *Weinmann, Meyer, Mardfin & Weiss* 1959, *Fasske & Morgenroth* 1962, *Mori, Mizushima & Koizumi* 1962, *Quintarelli* 1962 and *Ten Cate* 1963) Other investigations compare histochemically the non-keratinized normal buccal mucosa with pathological specimens (*Svoboda, Lojda & Skach* 1959, *Gerson & Meyer* 1964) *Meyer & Gerson* (1964) compared the morphology and other aspects of the buccal mucosa and of the keratinized mucosa of the palate *Haim* (1964) has studied the buccal mucosa with the periodic acid Schiff method

The ultrastructure of keratinized buccal mucosa of mice has been studied by *Sognnæs & Albright* (1956), who have also reported on a preliminary study of the human buccal mucosa (1958) *Themann* (1958) has reported some ultrastructural differences between normal and carcinomatuos mucosa In 1959, *Fasske & Themann* concluded that the oral mucosa is not a syncytium and that the junction between the connective tissue and the epithelium is formed by two membranes Ultrastructural aspects of non keratinizing human buccal mucosa have been studied by *Zelickson & Hartman* (1962) and by *Haim* (1964)

The aim of the present investigation is to relate some histochemical aspects of the human, non keratinized buccal mucosa with its ultrastructure

MATERIAL AND METHODS

Histochemistry

The material consisted of 10 biopsies of check mucosa from six females and from four males The biopsies were taken with a 5 mm punch under local anaesthesia Only persons that did not suffer from diseases affecting the oral mucosa were in

[illegible]

cluded Their age varied from 41 to 68 years After removal the tissue was bisected with crossed razor blades One half was fixed in neutral formalin, brought to paraffin and sectioned at 6 µ The other half was immersed in isopentane cooled with liquid nitrogen and sectioned at 10 µ on a SLEE cryostat microtome

The cryostat sections were incubated to demonstrate dehydrogenases and hydrolytic enzymes For dehydrogenases the following stock solution was used

Nitro BT (Sigma) 1 0 mg/ml	0 25 ml
Phosphate buffer, 0 2 M, pH 7 2	0 25 ml
Distilled water	0 40 ml

To this stock solution one of the following substrates was added

For *DPNH diaphorase* 2 mg of DPNH (Sigma) dissolved in 0 1 ml of dist water

For *Succinic acid dehydrogenase* 0 1 ml of M disodium succinate (Fluka)

For *Glucose-6-phosphate dehydrogenase* 0 1 ml 1 0 M dipotassium glucose 6 phosphate (Sigma) with 2 mg of DPN (Sigma) as coenzyme

The time of incubation was in all cases 30 minutes, and the temperature 37° C. Following incubation the sections were briefly rinsed with distilled water and post fixed in cold acetone for 5 minutes They were then washed in tap water, rinsed in distilled water and mounted in glycerol gelatine Control sections were treated similarly but incubated in medium without substrate

The hydrolytic enzymes investigated were acid phosphatase and alkaline phosphatase Acid phosphatase was demonstrated with the method of *Gomori* (1950) and that of *Barka & Anderson* (1963) For the demonstration of alkaline phosphatase the method of *Burstone* (1962) was used Controls were incubated without substrate Two sections of each biopsy were stained with haematoxylin eosin

Various histochemical procedures were performed on the paraffin sections They were examined for basophilia after staining with gallocyanin before and after treatment with ribonuclease (*Lison* 1960) Proteins were investigated by methods using naphtol yellow S (*Meek* 1962) and the ninhydrin Schiff reagent (*Lison* 1960) For basic proteins the method of *Wagner* (1955) was used Protein bound carboxyl groups were demonstrated with the method of *Barrnett & Seligman* (1958) and tryptophane by the method of *Adams* (1957) Protein bound sulphydryl and disulphide groups were demonstrated by the method of *Barrnett & Seligman* (1954) Reduction of disulphide groups was performed with 0 5 M thioglycollic acid for 4 hours at 37° C (*Barrnett & Seligman* 1954)

Carbohydrates were detected with the periodic acid Schiff (PAS) method before

(*Lison* 1960)

Electron Microscopy

The material consisted of 6 biopsies The same biopsy technique as previously

the tissue was reoriented so as to place the plane of sectioning at right angle surface of the tis ... were based on an examination of 1 ... & Winchester 1963) The blocks ... in thickness of 600 to 800 Å The ... with polyvinyl and a carbon film ... and examined in a Siemens Elmiskop I

RESULTS

A *Histological Observations*

In evaluating the sections the epithelium was divided into three layers, a stratum basale consisting of cells in contact with the base-

ment membrane a stratum spinosum beginning at the cell layer next to the basal layer, comprising most of the epithelium and a surface layer The stratum spinosum was subdivided into three parts a basal part an intermediary part and a superficial part There is no distinct borderline between the surface layer and the stratum spinosum in the non keratinized epithelium where the surface layer consists of the most superficial two to three cell layers whereas there is a marked difference between the two layers when keratinization occurs In the light microscope two biopsies demonstrated a slight parakeratosis characterized by a surface layer two to three cell layers thick showing eosinophilia and pycnotic nuclei The most superficial part of the stratum spinosum showed enlarged cells with a clear cytoplasm These cells were rare in the non keratinized mucosa where the cells are flattened In the remaining part of the epithelium it was not possible to distinguish between a non keratinized and a slightly parakeratinized mucosa

B *Histochemical Observations*

Dehydrogenases Succinic acid dehydrogenase and glucose 6-phosphate dehydrogenase demonstrated the greatest activity in the basal layer decreasing activity outwards through the stratum spinosum and a low activity or no activity at all in the surface layer The activity of DPNH diaphorase was greatest in the basal layer In the basal part of stratum spinosum the activity was markedly reduced and this level was retained throughout the layer At the surface layer there was a slight decrease The control sections showed no deposition of formazan

Phosphatases The activity of acid phosphatase as demonstrated with the method of *Barka & Anderson* was low in the basal layer increasing in the basal part of the stratum spinosum and culminating in the intermediate part thereafter decreasing again towards the surface layer where it disappeared (Fig 1) In two biopsies demonstrating a slight parakeratosis the activity of acid phosphatase was highest just below the surface layer (Fig 2) Visualization of acid phosphatase using the method of Gomori gave very inconsistent results

Alkaline phosphatase activity could not be demonstrated in the epithelium but was very intense in the endothelial cells of the underlying capillaries

Basophilia The most intense basophilia was found in the cytoplasm of the basal cells The intensity of the stain decreased gradually throughout the stratum spinosum and was very weak in the surface layer Sections treated with RNase before staining showed a staining of the nuclei only

Proteins Treatment with ninhydrin Schiff and naphtol yellow S gave an intense colour reaction in the cytoplasm of the basal cells a somewhat weaker staining of the stratum spinosum and again an





Figs 1 2

Difference in distribution of acid phosphatase in non keratinized (Fig 1) and para keratinized (Fig 2) buccal mucosa Barka method on fixed tissue naphthol AS BI as substrate × 121

intense staining of the surface layer The protein bound carboxyl groups showed a similar distribution but the difference in staining intensity between the three layers was more pronounced The reaction for tryptophane was weak in the stratum basale and the stratum spinosum but more pronounced in the surface layer Staining with chromotrope 2R for basic proteins resulted in a positive reaction in nucleoli of some basal cells and in nuclei of the surface layer The



Fig 3

Basal layer of non keratinized buccal mucosa The basement membrane (bm) has an irregular course, the contact with the basal cells is formed by "half" desmosomes (hd) Several mitochondria (m), tonofilaments (tf) and ribosomes (r) are seen × 17500

DDD reaction for -SH groups gave a moderate staining of the basal layer, a weaker staining of the stratum spinosum and almost no staining or no staining at all in the surface layer After reduction of the -SS- groups with thioglycollic acid the stratum basale and the stratum spinosum retained their staining intensity, while the staining of the surface layer was increased to the same level as in the basal layer

Carbohydrates The periodic acid-Schiff reaction showed no glycogen in the basal layer The basal part of the stratum spinosum contained a little glycogen and the content increased up to the surface layer where it was the highest Sections pretreated with diastase demonstrated diastase resistant PAS-positive material in the basement membrane, a low content of positive material in relation to the cell boundaries in the stratum spinosum and an increase in the surface layer with the same localization The three methods for metachromasia showed no metachromatical material in the epithelium Staining with alcian blue was not informative as it gave a uniform staining of the entire epithelium

C. *Electronmicroscopical Observations*

Stratum basale The basal layer was one to two cell layers in thickness (Fig 3). It consisted of elongated cells, 15 20 μ in length, oriented





Figs 4–5

Fig 4 Part of a basal cell showing "half" desmosomes (hd) Notice the lines (arrow) in the space between the basement membrane (bm) and the basal cell (bc) × 40000

Fig 5 Cells from the basal part of the stratum spinosum In the cytoplasm mitochondria (m) are scattered and two Golgi zones (gz) are present The endoplasmic reticulum is present as rough surfaced vesicles (er) (ds) desmosome × 17500

perpendicular to the basement membrane The cell surface was folded with extensions forming a zipperlike system with that of the opposing cell Contact between apposing cells was formed by numerous desmosomes, approximately 0 25 μ in length At the point of contact with the basement membrane a thickening of the cell membrane was formed which together with the central disc of a desmosome formed a "half desmosome" (Fig 4) The basement membrane was thickened corresponding to the "half desmosome" The space between the basement membrane and the central disc seemed to be filled with an amorphous substance In the basal cell, filaments were seen attached to the thickening of the cell membrane, and in the connective tissue filaments, approximately half the diameter of a collagen fiber, were attached to the basement membrane In one section a monocyte was seen, in other sections a few lymphocytes were seen to penetrate between the basal cells

The cytoplasm of the basal cells contained many small mitochondria, which were distributed polarly to the nucleus The Golgi apparatus was usually found apically to the nucleus but was small of size Ribosomes were abundant in the cytoplasm, whereas endoplasmic reticulum was limited to a few rough-surfaced vacuoles The cytoplasm contained numerous filaments lying either singly or in bundles with a random orientation The thickness of a filament was approximately 80 Å The nucleus was relatively large Mitosis was not seen

Stratum spinosum The cells in the stratum spinosum exhibited great variability In the basal part of the layer, they had almost the same appearance as the basal cells (Fig 5) They were rounded or oval in shape with a folded surface Contact with adjacent cells consisted of numerous desmosomes of approximately the same size as those in the basal layer The cytoplasm was electron dense It contained several mitochondria and a sparsely developed endoplasmic reticulum which presented elongated rough surfaced vacuoles The ribosomes were predominantly free, occuring either singly or in clusters Most of the density of the cytoplasm was due to the presence of filaments or bundles of filaments Apart from their relation to the desmosomes the filaments had no obvious orientation

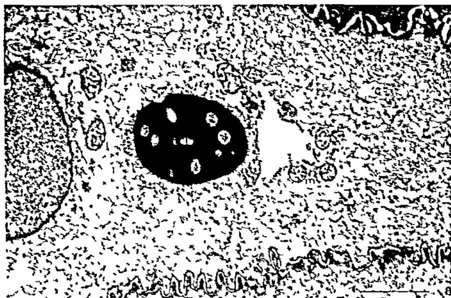
In the intermediate part of the stratum spinosum the orientation of the cells changed so that the long axis of the cells became oriented parallel to the surface of the epithelium (Fig 6) The surface of the cells was still heavily folded but the extensions were shorter The desmosomes were less numerous and smaller than in the basal layer The intercellular space had become narrower and had a width of approximately 0 5 μ The distribution of the mitochondria was in accordance with the altered orientation of the cells so that they retained their distribution to the sides of the nucleus The endoplasmic reticulum continued to be sparse and the free ribosomes abundant The vacuoles forming the endoplasmic reticulum were situated in the perinuclear area Only a few





Fig 6 Part of a c [illegible] part of stratum spinosum The intercellular space [illegible] and *bridged by several desmosomes (ds) The tono* filaments are dispersed in the cytoplasm (tf) leaving a narrow zone ar und *the nucleus (nu) free Ribosomes are seen mostly in clusters (r) (m) mito*chondria × 17500

Fig 7 Part of a "dense cell" in the upper stratum spinosum *The amorphous* material (ma) possibly represent a remnant of the nucleus Notice the thick bundles of filamentous material × 35000





Figs 8-9

Fig 8 'Dense body" (db) in the upper part of stratum spinosum The body has a minor indentation and some granulated areas or invaginations A cloud of particles surrounds the body In upper right corner, part of a dense cell is seen The mitochondria (m) show disintegration Tonofilaments are present but are small and few × 35000

Fig 9 Surface layer and upper part of stratum spinosum of a non keratinized specimen The cytoplasm shows almost no fibrillar content, desmosomes (ds) are small and few The mitochondria (m) show disintegration and the nuclei (nu) are decayed (es) epithelial surface × 17500

single filaments were present in this area In the remaining cytoplasm numerous filaments were *forming a network with the ribosomes interspersed* No structures were found that could be identified as lysosomes The nucleus was relatively big and round with minor identations

In the outer part of the stratum spinosum the cells became more elongated (Fig 9) *The cell membrane was still folded but the desmosomes* showed a further decrease in size and number. The cytoplasm was less dense and contained only a few mitochondria showing signs of degeneration The endoplasmic reticulum had almost disappeared *It was limited to one or two rough-surfaced vacuoles* The number of ribosomes had also decreased They were sometimes seen in close connection to the filaments These were fewer and thinner, approximately 40A in diameter, furthermore shorter and more frequently *single without obvious orientation* The cells also contained varying areas of cytoplasm without structures

In the same part of the stratum spinosum, cells with dense cytoplasm were seen (Fig 7) These cells were about 1 μ wide and 30–40 μ in length Sometimes they were lying in continuation of one another with an overlapping zone at their junction The middle of the cell was about 4 μ wide and contained a material that might represent the nucleus It was not possible to distinguish organelles in the remaining cytoplasm because of a dense network of filaments

In some biopsies, bodies of a high electron density were seen in a few cells neighbouring the dense cells (Fig 8) The bodies had sizes ranging from 0 5 μ to 2 μ The smaller ones were almost circular and were quite homogeneous, the larger ones were of oval shape except for an indentation In some of the larger bodies the central area was slightly granulated, while others contained some small granulated areas The bodies were surrounded by a cloud of particles that might be identified as ribosomes Close to the bodies a structureless area of cytoplasm was frequently observed, in some places limited by rows of ribosomes The electron density of the bodies was different from that of keratohyalin and indicated a content of metal

The surface layer Based on light microscopy four specimens were non-keratinized, the only difference between the surface layer and the upper part of stratum spinosum being a minor enlargement of the cells Two specimens demonstrated a parakeratosis characterized by a heavy staining of the surface layer with toluidine blue *Keratohyalin granules* were not evident in the sections

The surface layers of the four biopsies showing a non keratinized surface were alike The layer was 1 to 2 cell layers thick (Fig 9) The cells were elongated, measuring approximately *3 μ × 20 27 μ* The surface facing the oral cavity was slightly folded The surface facing the underlying cells was more folded but the folds of adjacent cells interdigitated only to a minor degree This in conjunction with sparse and small desmosomes gave the cells a *loose contact with each other* The



Fig 10

Upper part of stratum spinosum and surface layer of a parakeratotic specimen The "root system" (rs) consists of dense cells (c1) and "spongy" cells (c2) (es) epithelial surface (am) amorphous material with vacuoles × 17500

cytoplasm was more electron dense than that of underlying spinous cells Organelles could not be seen except for some remnants of mitochondria The number of filaments had decreased further The electron-dense material contained no structures The nucleus was present but showed signs of degeneration

One of the parakeratotic biopsies revealed two types of surface layer One type was formed of 6 to 7 cell layers (Fig 10) The superficial three layers consisted of cells of a thickness of 0 5 μ The cell surface facing the underlying cells was folded but desmosomes could not be recognized, no remnants of nuclei or organelles could be seen as the cell was filled with a dense material The underlying 3 to 4 cell layers consisted of two types of cells, one of which was rather electron dense These sent processes down between the underlying spinous cells, thus forming a kind of "root system" The surface of the "roots" was deeply folded with several long extensions of the cytoplasm The desmosomes were sparse and small In the cytoplasm organelles could not be seen but material of varying density was present either in dense amorphous clumps or as less dense areas containing numerous embedded filaments In the former type vacuoles were often seen in relation to the material, in the latter type vacuoles were not present The second type of cells also had a deeply folded surface Organelles were not present Part of the cytoplasm contained dense material related to vacuoles but the rest of the cytoplasm was filled with a coarse material that gave the cell a spongy appearance

In the second type of surface the difference between the surface cells and the cells of the stratum spinosum was not as pronounced There was no "root system" The surface of the cells was rather folded and demonstrated only a few indistinct desmosomes The electron density of the cells was unchanged from that in the spinous cells The cytoplasm showed loss of organelles In some instances it contained vacuoles to which an amorphous substance was attached The filaments seemed to have disappeared and the cytoplasm was of a low density The nucleus was decayed The density was increased in the outermost cells These showed no organelles but only the cell membrane The surface facing the oral cavity was smooth

The second parakeratotic biopsy showed a surface where the "root system" was either poorly developed or absent The cells in the layer above the enlarged cells of the stratum spinosum were emptier as the filaments seemed gradually to be transformed into a amorphous material In the following cell layer this material was more coarse giving the cells a spongy appearance In the outermost cells the density of the cells was markedly increased and they seemed to be filled with a mixture of filaments and amorphous material In all layers the cell membrane was folded and formed only a few small indistinct desmosomes The boundary between the cells could be followed almost to the surface where it was obscured by a blood clot In none of the paraker-

totic specimens were keratohyalin granules seen with the electr
microscope

DISCUSSION

The potential for aerobic production of energy seems to decrease alm
continuously throughout the epithelium The activity of succinic a
dehydrogenase is highest in the basal cells, as is the number of mi
chondria Both decrease towards the surface, and where the activ
of the enzyme disappears completely, the mitochondria show sig
of disintegration Likewise the energy formation through the pent
shunt seems to disappear gradually since the activity of glucose
phosphate dehydrogenase gradually disappears Thus the amount
energy potentially available in the surface layer appears alm
negligible

The hydrolytic enzyme acid phosphatase has been shown by de *Du*
(1955) to be one of the enzymes contained in the cytoplasmic partic
called lysosomes In the present study the highest activity of ac
phosphatase was demonstrated in the upper part of the stratum s
nosum but it was not possible to demonstrate lysosomes in the electr
microscope This might simply be caused by the absence of lysosom
Epithelial cells in the sections used for histochemical visualization
acid phosphatase with the method of *Barka & Anderson* did not show
particulate but a uniform staining On the other hand, cells in t
connective tissue gave the same diffuse staining although one shou
expect a particulate staining of the lysosomes in the histiocytes U
fixed fresh-frozen sections are probably not optimal for the demo
stration of lysosomes Staining for acid phosphatase with the method
Barka & Anderson, and using naphtol AS-BI phosphate as substra
was therefore performed on sections fixed in cold formalin CaC
embedded in gumsucrose These sections showed distinct granules
the histiocytes in the connective tissue but the same diffuse staini
in the epithelial cells as did the unfixed sections This supports t
view, that the acid phosphatase in oral epithelium is not present
lysosomes This conclusion is in contrast to that of *Ten Cate* (196
Ten Cate found that the activity of acid phosphatase of non keratiniz
oral mucosa in stratum germinativum and stratum spinosum was loc
ized to small granules distributed mainly at the cell periphery a
perinuclearly In the most superficial cells the granules were larg
and distributed irregularly throughout the cell *Ten Cate* related the
granules to lysosomes The contrast to the findings of the present stu
may be explained by the fact that *Ten Cate* used the method of Gomo
on unfixed frozen sections which according to *Holt* (1959), is n
optimal

The results concerning alkaline phosphatase activity are in agre
ment with those of *Cabrini & Carranza* (1951) and of *Zander* (1941

When alkaline phosphatase activity is found in the oral epithelium it may be due to a local disorder (*Montagna & Elles* 1962)

In keratinized epithelium two types of protein related to keratin are found, one as the fibrillar tonofilaments and the other as the amorphous material represented by keratohyalin granules In the present study keratohyalin granules were not found, neither in the non-keratinized specimens nor in the parakeratotic specimens, whereas the tonofilaments were found in all cells in a smaller amount (*Brody* 1960 *Charles & Smuddy* 1957) Corresponding to the finding that the amount of filaments was highest in the basal layer, relatively strong staining with naphtol yellow S and staining indicative of protein bound carboxyl groups was found here, together with moderate staining in the ninhydrin-Schiff procedure and a moderate reaction for —SS— and —SH groups In the stratum spinosum the intensity of the above mentioned reactions decreased, corresponding to the decrease in amount of filaments In the surface layer, however, the intensity of staining with ninhydrin-Schiff, naphtol yellow S and for carboxyl groups all increased and furthermore the content of cystine seemed to rise as did the content of tryptophane The significance of this change is not clear The ultrastructural findings did not show an increase in the amount of fibrillar components but it showed an increase in electron density in the most superficial cells and a progressive decrease in number of ribosomes throughout the epithelium Furthermore, the nucleoproteins showed disintegration as the basic proteins in the nuclei of the most superficial cells reacted with chromotrope 2R It therefore seems unlikely that the increase in stainability and electron density should be caused by protein synthesis rather by the degeneration of the cells

It is difficult to give a satisfactory reason for the high content of glycogen in the surface layer The most acceptable explanation seems to be that the glycogen represents an excess not utilized as is the case in keratinized oral epithelium, where glycogen cannot be demonstrated (*Weinmann et al* 1959, *Gerson & Meyer* 1964, *Bulow, Clausen & Renstrup*) Other investigators (*Montagna* 1962, *Wislocki et al* 1951) have reported the same findings, whereas *Haim* (1964) reports a lack of glycogen in non keratinized oral mucosa, which he relates to the lack of keratinization An evaluation of his findings is impossible as the author does not report of any control of the staining with the PAS method

The surface layer of the non keratinized epithelium contained a PAS-positive, diastase-resistant material also described by *Weinmann et al* (1959) and *Wislocki et al* (1951) *Weinmann et al* found this material to be deposited between the cells and to have a beaded appearance due to the interdigitation of the cell membranes *Wislocki et al* found the material to be deposited partly as granules related to the cell membrane and partly as a homogenous material in the intercellular

pace Granules were not seen in the present study It was not possible o establish the exact localization of the homogenous material In elec ron microscopic preparations the intercellular spaces are seen to be ery narrow approximately 0 1 to 0 5 μ in width and thus at the limit f the resolving power of the light microscope It may be therefore hat the intercellular material is attached to the cell membrane and epresents the glycocalyx described by *Bennett* (1963)

In the electron microscope the biopsies demonstrating a parakera osis were found to have a surface layer thicker than that of non kera inized mucosa and a root system anchoring the superficial cells to he underlying cells However there were no keratohyalin granules present and the underlying cell layers did not differ markedly from hose of non keratinized mucosa This seems to indicate that the non keratinizing oral mucosa pocesses the ability to keratinize in a way different from the one found in orthokeratosis or in parakeratosis with a stratum granulosum The single dense cells in the upper part of tratum spinosum may be regarded as an intermediate stage

The only histochemical difference between the non keratinized and the parakeratinized mucosa was shown by the distribution of acid phosphatase As the role of acid phosphatase in the epithelium is not known this difference only indicates that there is a relation between the activity of the enzyme and the process of keratinization

SUMMARY

Histochemical and fine structural aspects of the non keratinized buccal mucosa are presented Both the histochemical and the ultrastructural findings show that the amount of energy potentially available is de creasing throughout the mucosa The localization of acid phosphatase in the non keratinized mucosa is different from the localization in the case of parakeratosis without a stratum granulosum Evidence of a non lysosomal localization is given Alkaline phosphatase could not be demonstrated in the mucosa In the non keratinized mucosa there is no synthesis of protein in the superficial cell layers but the disintegration of the cells gives rise to an increased stainability and electron density Abundant glycogen is found in the superficial cell layers possibly representing an excess not utilized as it is in keratinized epithelium

Although all the biopsies were regarded as being clinically non keratinized the material contained besides non keratinized mucosa two biopsies demonstrating parakeratosis without a stratum gran ulosum Nevertheless the basal cells the lower and the middle part of the stratum spinosum had the same appearance in all biopsies the changes being confined to the surface layer only The parakeratotic surface layer was characterized by the formation of eosinophilic cell layers 2 to 3 cell layers thick with pycnotic nuclei In the electron

microscope it appeared to consist of 6 to 7 cell layers the most superficial ones being extremely thin, and anchored to the underlying cells by a ' root system Keratohyalin granules were not present

REFERENCES

Adams C W M A p dimethylaminobenzaldehyde nitrite method for the histochemical demonstration of tryptophane and related compounds J Clin Path *10* 56–62 1957

Barka T & Anderson P J Histochemistry Theory Practice and Bibliography Hoeber Medical Division Harper and Row Publ New York 1963

Barrnett R J & Seligman A M Histochemical demonstration of sulfhydryl and disulphide groups of proteins J Nat Cancer Inst *14* 769–803 1954

Barrnett R J & Seligman A M Histochemical demonstration of protein bound alpha acylamide carboxyl groups J Biophys Biochem Cytol *4* 169 176 1958

Bennett H S Morphological aspects of extracellular polysaccharides J Histochem Cytochem *11* 14 23 1963

Bennett H S & Luft J H s collidine as a basis for buffering fixatives J Biophys Biochem Cytol *6* 113 114 1959

Brody I The ultrastructure of the tonofibrils in the keratinization process of the normal human epidermis J Ultrastr Res *4* 264 1960

Burstone M S Enzyme histochemistry and its application in the study of neoplasms Academic Press New York 1962

von Bulow F A Clausen F P & Renstrup G in preparation

Cabrini R L & Carranza F A Histochemical study on alkaline phosphatase in normal gingivae varying the pH and the substrate J Dent Res *30* 28 1951

Charles A & Smiddy F G The tonofibrils of the human epidermis J Invest Dermat *29* 327 338 1957

de Duve C The lysosome concept In Lysosomes Ciba Foundation Symposium J & A Churchill London 1963

Fasske F & Themann H Über das Deckepithel der menschlichen Mundschleimhaut Licht und Elektronenmikroskopische Untersuchungen Zeit Zellforsch *49* 447 463 1959

Fasske E & Morgenroth K Histotopochemische Enzymmuster in der menschlichen Mundhöhle Deutsche Zahnarzteblatt *16* 333 341 1962

Gerson S J & Meyer J Metaplastic keratinization in the human buccal mucosa J Dent Res *43* 536 547 1964

Gomori G An improved histochemical technique for acid phosphatase Stain Tech *25* 81 85 1950

Haim G Elektronenmikroskopische Untersuchungen des normalen Epithels der menschlichen Mundschleimhaut Carl Hanser Verlag München 1964

Holt S J Factors governing the validity of staining methods for enzymes and their bearing upon the Gomori acid phosphatase technique Exper Cell Res suppl *7* 1 27 1959

Ito S & Winchester R J The fine structure of the gastric mucosa in the bat J Cell Biol *16* 541 577 1963

Kramer H & Windrum G M The metachromatic staining reaction J Histochem Cytochem *3* 227 237 1955

Lison L Histochimie et Cytochimie animales Gauthier Villars Paris 1960

Meek E S The cytochemical estimation of protein using naphtol yellow S and combined measurement of deoxyribonucleic acid J Histochem Cytochem *10* 564–567 1962

Meyer J & Gerson S J A comparison of human palatal and buccal mucosa Periodontics 2 284–291 1964

Montagna W & Ellis R A Histochemistry of enzymes in the skin Handbuch der Histochemie 7 39 72 1962

Mori M Miushima T & Kobayashi K A comparative histochemical evaluation of various dehydrogenases in the oral squamous epithelium Histochemie 3 111 121 1962

Quintarelli G Histochemistry of mucous membranes Ann *Histochim* 7 47 50, 1962

Sognnæs R F & Albright J T Preliminary observations on the fine structure of oral mucosa Anat Rec *126* 225–240 1956
[illegible] the
[illegible] of
Ten Cate A R The distribution of acid phosphatase non specific esterase and lipid in oral epithelia in man and the macaque monkey Arch Oral Biol *8* 747 755 1963
Theman H Elektronenmikroskopische Untersuchungen der normalen und der patologisch veranderten Mundschleimhaut Fortschr Kiefer und Gesichtskir *4* 390–398 1958
Wagner B M Histochemical studies of fibrinoid and other abnormal tissue proteins 1 Application of Roque s chromotrope stain A M A Arch Path *59* 58 1955
Weinmann J P Meyer J Mardfin D & Weiss M Occurrence and role of glycogen in the epithelium of the alveolar mucosa and the attached gingiva Am J Anat *104* 381 402 1959
Wislocki G B Fawcett D W & Dempsey E W Staining of stratified squamous epithelium of mucous membranes and skin of man and monkey by the periodic acid Schiff method Anat Rec *110* 359 1951
Zander H A The distribution of phosphatases in gingival tissue J Dent Res *20* 347 353 1941
Zelickson A S Electron microscopy of skin and mucous membrane Charles C Thomas Publ Springfield Ill 1963
Zelickson A S & Hartmann J F An electron microscope study of normal human non keratinizing oral mucosa J Invest Dermat *38* 99 1962

Acta path et microbiol scandinav 66 426–436 1966

Cancer Research Institute (Head Professor J Bichel M D) The Radium Centre Aarhus Department of Neurology (Head E Pedersen M D) Kommunehospitalet Aarhus, and Neurochemical Research Unit (Head M Schou M D) Risskov, Denmark.

THE LACTATE DEHYDROGENASE ISOENZYMES IN VARIOUS ORGANS OF THE RABBIT IN ANAEMIA, HYPOXIA, AND AFTER COBALT ADMINISTRATION

With Special Reference to Changes in the Isoenzyme Pattern of the Kidney Cortex

By

EIVIND B THORLING and KNUD JENSEN

Received 12 ix 65

In a previous study lactate dehydrogenase (LDH) isoenzyme patterns were studied in different parts of the kidney of normal rabbits compared with rabbits with a severe anaemia due to copious bleeding and after administration of a large dose of cobalt (*Jensen & Thorling* 1965) It was shown that the LDH isoenzyme pattern was changed, most constantly in the cortex of the kidney, revealing an increase in the most cathodic fraction, isoenzyme 5 It was suggested that these changes were due to hypoxia

In the present work these observations are confirmed by further analyses applying a new technique (*Jensen* 1965)

If the changes observed in the LDH isoenzyme pattern could really be ascribed to hypoxia, it would perhaps be possible to demonstrate similar changes in the kidney of rabbits submitted to restricted oxygen supply Our investigations were therefore extended to include animals kept at an atmosphere containing 10 per cent oxygen and 90 per cent nitrogen at ambient pressure for 24 hours

Furthermore, analyses were performed on liver, heart skeletal muscle cerebrospinal fluid, serum and various parts of the brain

---

The investigation has been supported by grants from *Carlsbergfondet Kong Christian den Tiendes Fond F L Smidth & Co A S s Jubilæumsfond P Carl Petersens Fond The Danish Anti Cancer League* and *The Danish League against Multiple Sclerosis*

The authors wish to express their gratitude for skilled technical assistance to Mrs *Else Poulsen* Mrs *Jette Lyberth* and Mrs *[illegible] Stougaard* and to Mr *Jens Vendelbo* M D in Economics for his advice regarding the statistical calculations

MATERIAL AND METHODS

The studies were performed on materials obtained from adult rabbits of both sexes. Only rabbits in which the haematocrit value was above 34 per cent at the beginning of the assay were included. The rabbits were studied in 4 groups:

I. *Normal rabbits* (11 animals).
II. *Anaemic rabbits* (6 animals). The anaemia was provoked by daily bleeding for 5 days 30–40 ml a day. The blood was taken from an ear vein. Only rabbits with a final haematocrit value at 20 per cent and below have been included.
III. *Hypoxic rabbits* (9 animals). The rabbit cage was placed in a large plast bag. A mixture of 10 per cent oxygen and 90 per cent nitrogen was passed through the bag with a flow of 4–6 litres per minute for 24 hours.
IV. *Rabbits challenged with Cobalt* (2 animals). 20 mg of Cobalt (as chloride in 15 ml saline) were injected subcutaneously. The rabbits were killed 24 hours later.

The rabbits were killed by insufflation of 30 ml air in an ear vein.

Preparation of the Tissues for Examination

Kidney. The kidney was cut from pole to pole in slices about 3 mm thick. A slice containing cortex and the pyramid including the apex was chosen. From this slice samples were taken in sequence from apex to cortex. Sample 1 represents the apex, 2 and 3 the medullary substance, sample 4 and 5 the cortex (Fig. 1).
Heart muscle. A sample was taken from the apex of the left ventricle.
Skeletal muscle. The sample was obtained from the psoas muscle.
Liver. The sample was usually taken from the centre of the right lobe.
Brain. Samples were taken from cortex of the frontal lobe, thalamus, cortex of the cerebellar vermis, superior and inferior colliculus of the mesencephalon and

the investigation.
Serum. Blood was collected from the caval vein and centrifuged immediately after coagulation.
Cerebrospinal fluid was taken by suboccipital puncture and centrifuged immediately to avoid contamination from cells. Blood tinged specimens were discharged.
The cerebrospinal fluid proteins was concentrated to about 1/100 of the original volume by vacuum dialysis in an apparatus supplied by Membranfilter Gesellschaft, Gottingen, Western Germany.

Polyacrylamide Gel Electrophoresis, LDH Isoenzyme Determination and Photometry

The electrophoresis was performed in a 4 per cent polyacrylamide gel prepared in a barbital buffer p [illegible] minutes at 250 V, 80 mAmp [illegible] electrophoresis apparatus.
LDH isoenzyme [illegible] van der Helm et al. (1962) and scanning performed in a Chromoscan densitometer at 595 nm.
To each electrophoresis 5 µl of the supernatant were used. The electrophoresis was performed twice for all samples and each figure provided in the tables represents the average of the two determinations.

RESULTS

In our previous work separation of the isoenzymes was carried out in an agar gel. At the end of the study however we ran into insurmountable trouble on account of, as proved later, a new batch of agar in

which the separation of the isoenzymes was unsatisfactory, especially concerning the separation of isoenzymes 4 and 5 As to our experience the polyacrylamide gel had at that time turned up to be more reliable, we decided to use this gel as a supporting medium in our further studies The details of a comparison of the two methods are given elsewhere (*Jensen* 1963, 1965).

According to the subunit theory by *Appella & Markert* (1961), *Cahn et al* (1962) and *Markert* (1963a) the five LDH isoenzymes are made up by 2 subunits "H" and "M". Each isoenzyme contains 4 of these subunits giving the following 5 possible constitutions of the isoenzymes



In the present work the "total M" value is calculated from the following formula.

$$M = \frac{(MMMM) \times 4 + (HMMM) \times 3 + (HHMM) \times 2 + (HHHM)}{4}$$

(The brackets indicate the percentage of the respective isoenzyme).

This enables us to give a single figure for each sample instead of one for each of the five isoenzymes

Consequently, the H value is expressed by the formula:

$$H = 100 - M$$

Changes in the content of M component were most obviously seen in the 5th, most cathodic isoenzyme and we would have reached the same conclusion of our study by using the 5th fraction alone

Normal Rabbits

The mean values obtained in normal rabbits are listed in Table I In Fig 1 the M values are shown for various organs of the normal rabbit

It is obvious that the M component dominates in the samples from skeletal muscle (92 5 per cent), whereas in the heart muscle only a small amount is demonstrable (6 1 per cent) This is consistent with works previously published in this field and justifies the use of the name "M" (muscle) for the "anaerobic" subunit and "H" (heart) for the "aerobic" component

The high M value found in the liver samples is also in good agreement with observations published by *Pfleiderer & Wachsmuth* (1961) and in accordance with the theory that the M component is characteristic for tissues with a low oxygen tension (*Fine et al* 1963)

The M values in the different zones of the kidney are of special interest as the kidney tissue has often been referred to as one "homogene" tissue concerning the LDH isoenzyme pattern (*Pfleiderer & Wachsmuth* 1961 and *Markert* 1962). Only few works have been pub-

TABLE 1

The Mean M Values of Various Organs Cerebrospinal Fluid and Serum of Normal Rabbits

	No of animals	Mean value of M %
Kidney apex I	11	53 9
– medulla II	11	43 0
– medulla III	11	31 1
– cortex IV	11	19 9
– cortex V	11	21 1
Heart	11	6 1
Muscle (psoas)	10	92 5
Liver	9	62 0
Cerebellar cortex	11	30 2
Frontal cortex	11	37 2
Chiasma	6	59 0
Colliculus sup	9	45 0
Colliculus inf	4	13 6
Thalamus	6	47 2
Cerebrospinal fluid	6	27 8
Serum	10	35 3

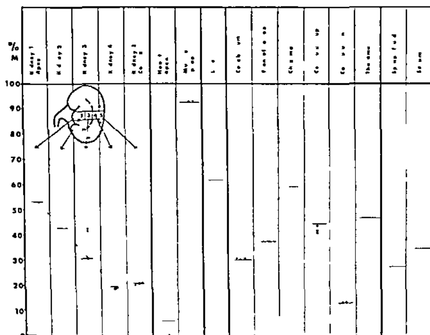


Fig 1

The M values of various organs cerebrospinal fluid and serum from normal rabbits The horizontal line in each column indicates the mean M value

lished in which it is clearly demonstrated that differences exist in the LDH isoenzyme pattern between the papilla and the cortex (*Richterich et al* 1961/62 *Fine et al* 1963, *Jensen & Thorling* 1965) It is seen from Fig 1 that the M component of the kidney tissue is decreasing from the apex (55 9 per cent) to the cortex (21 1 per cent) This difference is highly significant There is also a significant difference between the apex and zone 3 (31 1 per cent) which represents the most superficial part of the medulla (Table 2) These changes strongly support the theory of a correlation between the oxygen tension and the LDH iso enzyme pattern as various authors have established that the oxygen tension is considerably lower in the apex than in the cortex of the kidney (*Aukland & Krog* 1960 and *Ulfendahl* 1962)

TABLE 2

A Statistical Analysis of the Difference in M Values between the Apex of the Normal Rabbit Pyramid and the Outer Part of the Medulla (Zone 3) and between the Apex and the Superficial Part of the Cortex (Zone 5)

	No of animals	Mean value of M %	s	Difference	Students t	p
Apex 1	*11*	55 9	*9 3*	*0*		
Medulla III	11	31 1	8 8	24 8	6 0	p<0 001
Cortex V	11	21 1	3 8	34 8	10 9	p<0 001

The distribution of LDH isoenzymes has been studied in different regions of human brain (*van der Helm* 1962 *van der Helm et al* 1963 *Gerhardt et al* 1963a and *Lowenthal et al* 1964) and of ox brain (*Bonavita & Guarneri* 1963) and small differences were demonstrated In our present study (Fig 1 Table 1) the highest M value was found in the optic chiasma (59 0 per cent) which equals that of the liver and the papilla of the kidney This is in agreement with recent investigations by *Gerhardt* (1965) High values of M were also found in the thalamus (47 2 per cent) and the superior colliculus of the mesencephalon (45 0 per cent) this is in contrast to the inferior colliculus where the M value was only 13 6 per cent We found relatively high values in the frontal cortex and in the cortex of the cerebellar vermis (37 2 and 30 2 per cent respectively) which is in good agreement with *Gerhardt et al* (1963)

The M value of the cerebrospinal fluid was lower than in most brain tissues (*except for colliculus inferior*) and contamination from brain tissues is therefore unlikely The M values in serum were likewise usually higher than that of the cerebrospinal fluid from the same animals which militates against contamination from serum

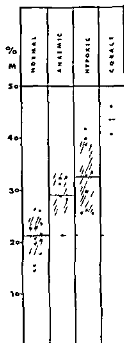


Fig 2

The mean M values of the most superficial part of the kidney cortex from normal anaemic, hypoxic and cobalt treated animals The horizontal line in each column indicates the mean M value, and the shaded area represents the standard deviation (s)

Treated Animals

In our previous work we demonstrated alterations in the isoenzyme pattern of the kidney cortex in animals made anaemic by copious bleeding and after cobalt administration These changes were interpreted as an enzyme-adaption to the relative hypoxia induced by the anaemia and the supposed "cellular hypoxia" induced by cobalt With the new polyacrylamide technique applied in the present investigation we were again able to demonstrate this shift in the isoenzyme pattern, expressed in this publication as an increase of the M component in the superficial part of the kidney cortex (Fig 2, Table 3) Furthermore this study includes a series of analyses performed on organs from rabbits submitted to isobar hypoxia In this group we could demonstrate a similar increase in the M component of the kidney cortex zone 5 The increase of the M component could be shown to be significant (using the "students t"-test) for the anaemic ($0.01 > p > 0.001$) and hypoxic ($p < 0.001$) rabbits Only two rabbits treated with cobalt are included in this study The M value for the kidney cortex of these animals was more than 5 times the standard deviation higher than the mean value in the normal group (Table 3 Fig 2) In zone 4 of the kidney the same

lished in which it is clearly demonstrated that differences exist in the LDH isoenzyme pattern between the papilla and the cortex (*Richterich et al* 1961/62 *Fine et al* 1963, *Jensen & Thorling* 1965) It is seen from Fig 1 that the M component of the kidney tissue is decreasing from the apex (55 9 per cent) to the cortex (21 1 per cent) This difference is highly significant There is also a significant difference between the apex and zone *3* (31 1 *per cent*) *which represents the most superficial* part of the medulla (Table 2) These changes strongly support the theory of a correlation between the oxygen tension and the LDH isoenzyme pattern as various authors have established that the oxygen tension is considerably lower in the apex than in the cortex of the kidney (*Aukland & Krog* 1960 and *Ulfendahl* 1962)

TABLE 2

A Statistical Analysis of the Difference in M Values between the Apex of the Normal Rabbit Pyramid and the Outer Part of the Medulla (Zone 3) and between the Apex and the Superficial Part of the Cortex (Zone 5)

	No of animals	Mean value of M %	s	Difference	Students t	P
Apex 1	11	55 9	9 3	0		
Medulla III	*11*	*31 1*	*8 8*	*24 8*	*6 0*	$p<0\,001$
Cortex V	11	21 1	3 8	34 8	10 9	$p<0\,001$

The distribution of LDH isoenzymes has been studied in different regions of human brain (*van der Helm* 1962 *van der Helm et al* 1963 *Gerhardt et al* 1963a and *Lowenthal et al* 1964), and of ox brain (*Bonavita & Guarneri* 1963) and small differences were demonstrated In our present study (Fig 1 Table 1) the highest M value was found in the optic chiasma (59 0 per cent) which equals that of the liver and *the papilla of the kidney This is in agreement with recent investigations by Gerhardt* (1965) High values of M were also found in the thalamus (47 2 per cent) and the superior colliculus of the mesencephalon (45 0 per cent) this is in contrast to the inferior colliculus where the M value was only 13 6 per cent We found relatively high values in the frontal cortex and in the cortex of the cerebellar vermis (37 2 and 30 2 per cent respectively) which is in good agreement with *Gerhardt et al* (1963)

The M value of the cerebrospinal fluid was lower than in most brain tissues (except for colliculus inferior) and contamination from brain tissues is therefore unlikely The M values in serum were likewise usually *higher than that of the cerebrospinal fluid* from the same animals, which militates against contamination from serum

under influence of hormones It is most likely that the ability to synthesize the M subunit was already present before the stimulation, and it should not be necessary to assume that the M component found in homogenates of the nonpregnant uterus should be derived from the connective tissue or vessels

Noteworthy is the fact that cells with a pronounced predominance of the H subunit *e g*, the tubulus cells of the kidney (*Richterich et al* 1961 62), may be transformed into malignant cells (hypernephroma) (*Oberling* 1959) in which the M subunit is the main component (*Pfleiderer & Wachsmuth* 1961)

Another question related to the former is whether the isoenzyme pattern characteristic of the tissues from adult animals is really as fixed as generally believed The demonstration of the myometrical change in isoenzyme pattern at least throws doubt on this assumption (*Richterich et al* 1963)

Our own investigations point in the same direction that the isoenzyme pattern of tissue from adult animals may under certain conditions be changed

Another, however less convincing, support for this theory is the investigations by *Güttler & Clausen* (1965), who found changes in the isoenzyme pattern of urine from kidneys with partial arterial occlusion It was presumed that the observed rise in the M component in the urine was due to hypoxic conditions in the kidney

It would have been of great interest to know whether the isoenzyme pattern of the hypophysis was changed in the hypoxic conditions provoked in our investigations, as it is known that the production of ACTH is considerably increased during hypoxia (*Marks et al* 1965) This was unfortunately not done Only one analysis was performed on one normal hypophysis revealing a low content of the M subunit (18 per cent)

However it must be stressed that changes were only observed in the kidney cortex and possibly in the liver as a response to hypoxia, and that the isoenzyme pattern was unaltered in all other tissues examined in this study

The third important problem in LDH research is whether a correlation exists between a specific isoenzyme pattern and the type of metabolism in the cells There seems to be good evidence of this theory originally stated by *Pfleiderer & Wachsmuth* (1961) The two parts of the theory may be expressed as follows

1 Predominance of the "H" subunit is correlated to high oxygen consumption, aerobic metabolism, and a highly differentiated mitochondrial apparatus

2 Predominance of the "M" subunit is correlated to low oxygen uptake, anaerobic metabolism with high glycolysis and less differentiated mitochondrial apparatus

This means too that tissues with a low oxygen supply are likely to

have a predominance of the M subunit in the build up of the isoenzyme pattern. The observations in our investigations support these hypotheses by confirming the observation previously made on the isoenzyme pattern *in various normal organs*. What is of special interest is that certain organs seem to be able to adapt in some degrees to changes in the oxygen supply by alterations in the isoenzyme pattern. It is interesting that *Guttler & Clausen* (1965) found a similar adaption to anaerobic conditions in tissue culture of kidney tissue.

The mechanisms involved in this enzyme adaption are obscure. It may be a matter of gene activation resembling the phenomenon observed by *Clever & Karlson* (1960) in the chromosomes of the larval Chironomus after application of ecdyson.

It would be extremely interesting to see whether changes in the isoenzyme pattern could be correlated to the malignant transformation observed in tissue cultures and in rats and mice after implantation of a plastfoil (*Alexander & Horning* 1959). In this last condition protracted local oxygen deficit has been proposed to be the carcinogenic factor. *Anyway it is known that malignant tissues contain predominantly the* M subunit even if they emerge from cells in which the H subunit is the main component. This means that malignant cells must acquire the ability to synthesize an increasing amount of M subunit or lose the mechanisms involved in the synthesis of the H subunit. It remains to be discussed whether the former or the latter of these suggestions is true.

SUMMARY

An investigation of the lactate dehydrogenase (LDH) isoenzyme pattern of various organs (listed in Table 1) of the rabbit is reported. The effect of anaemia and hypoxia on the isoenzyme pattern was studied. It was demonstrated that in the kidney cortex and presumably in the liver of the treated animals a change took place in the isoenzyme pattern. Based on this isoenzyme pattern the relative content of the M values was calculated. The changes observed include an increase in the relative content of the M subunit. In accordance with the M values found in normal tissues and the apparent correlation of a high M value to a *low oxygen tension the changes observed are interpreted as* an epigenetic adaption to the lowered oxygen supply imposed by the treatment. As previously reported we found a similar effect of high doses of cobalt which may be explained by the assumption that cobalt interferes with certain oxydation enzymes in the cells.

REFERENCES

Alexa [illegible] method of inducing
Ciba Foundation
G. E. W. Wolsten

Appella, E & Markert C L Dissociation of lactate dehydrogenase into subunits with guanidine hydrochloride Biochem Biophys Res Comm *6* 171 1961

Aukland K & Krog J Renal Oxygen Tension Nature *188* 671 1960

Bonavita V & Guarneri Rosa Lactate dehydrogenase isoenzymes in nervous tissue III Regional distribution in ox brain J Neurochem *10* 755 1963

Bonavita V Ponte F & Amore G Lactate dehydrogenase isoenzymes in the nervous tissue IV An ontogenetic study on the rat brain J Neurochem *11* 39 1964

Cahn R D Kaplan N O Levine L & Zwilling E Nature and development of lactic dehydrogenases The two major types of this enzyme form molecular hybrids which change in makeup during development Science *136* 962 1962

Clever U & Karlson P Induktion von Puff Veranderungen in den Speicheldrusen chromosomen von Chironomus Tentans durch Ecdyson Exp Cell Res *20* 623 1960

Fine I H., Kaplan N O & Kuftinec D Developmental changes of mammalian lactic dehydrogenases Biochemistry 2 116 1963

Gerhardt W Clausen J Christensen F & Rushede J Changes of LDH isozymes esterases acid phosphatases and proteins in malignant and benign human brain tumours Acta Neurol scand. *39* 85 1963 a

Gerhardt W Clausen J & Andersen H Electrophoretic patterns of extractable proteins and enzymes in embryonic and adult brains Acta Neurol scand *39* 31 1963 b

Gerhardt W & Andersen H Lactic dehydrogenases of brain Comparative studies of the visual pahtways Protides of the Biological Fluids Proceedings of the XIII Colloquium Bruges 1965 Ed H Peeters 173 178 Amsterdam 1966

Gutler F & Clausen J Urinary lactate dehydrogenase activity Determination of the urinary LDH isozyme pattern as a supplement to the measurement of total urinary LDH activity Enzymol biol clin 5 55–64 1965

Jensen K A comparison between polyacrylamide and agar gel as supporting media for electrophoresis Scand J Clin Lab Invest *15* suppl 76 73 1963

Jensen K & Thorling E B The effect of anaemia and cobalt on lactic dehydrogenase isoenzymes in kidney tissues of rabbits and its possible relation to the erythropoietin production Acta path et microbiol scandinav *63* 385 1965

Jensen K A simple method for protein electrophoresis in polyacrylamide gel Scand J Clin Lab Invest *17* 192 1965

Lowenthal A Karcher D & van Sande M Electrophoretic patterns of lactate dehydrogenase isoenzymes in nervous tissues J Neurochem *11* 247 1964

Markert C L & Møller F Multiple forms of enzymes tissue ontogenetic and species specific patterns Proc nat Acad Sci *45* 753 1959

Markert C L & Ursprung H The ontogeny of isozyme patterns of lactate dehydrogenase in the mouse Developm Biology 5 363 1962

Markert C L Developmental aspects of kidney form and function Isozymes in kidney development Hereditary devel pmental and immunologic aspects of kidney disease p 54 Northwestern University Press 1962 Ed Jack Metcoff

Markert C L. Lactate dehydrogenase isoenzymes Dissociation and recombination of subunits Science *140* 1329 1963 a

Markert C L Epigenic control of specific protein synthesis in differentiating cells Cyto differentiation and Macromolecular Synthesis Academic Press New York, 1963 b Ed M Locke

Marks B H Bhattacharya A K & Vernikos Danellis Joan Effect of hypoxia on *secretion of ACTH in the rat Amer J Physiol 208 1021 1965*

Oberling Ch Rivière M & Haguenau Fr Ultrastructure des épitheliomas à cellules claires du rein (hypernephromes ou tumeurs de Grawitz) et s n implication pour l histogenese de ces tumeurs Bull Cancer *46* 356 1959

Pfleiderer G & Wachsmuth E D Alters und funktionsabhangige Differenzierung der Lactatdehydrogenase menschlicher Organe Biochem *Z* 334 185 1961

Richterich R Schafroth P & Fran H E Das isolierte Glomerulum der Ratten niere III Heterogenitat der Lactat Dehydr genase in Nierenrinde Nierenmark und Glomerulum Enzym biol clin *1* 114 1961 62

Richterich R Schafroth P & Aebi H A study of lactic dehydrogenase isoenzyme pattern of human tissues by adsorption-elution of Sephadex DEAE Clin chim Acta *8* 178 1963

Ulfendahl H R Studies on the intrarenal separation of red cells and plasma Med Diss, Acta universitatis upsaliensis, Almqvist & Wiksell, Stockholm Sweden, 1962

van der Helm H J, Zondag H A Hartog H A Ph & van der Kooi M W Lactic dehydrogenase isoenzymes in myocardial infarction Clin chim Acta 7 540, 1962

van der Helm, H J Isoenzymes in different parts of the brain J Neurochem 9 325, 1962

van der Helm H J Zondag H A & Klein F On the source of lactic dehydrogenase in cerebrospinal fluid Clin chim Acta *8* 193, 1963

The Institute of Hygiene (Head Poul Bonnevie) and the Institute of Pathological Anatomy (Head G Teilum) of the University of Copenhagen Denmark

ON THE ULTRASTRUCTURE OF *NOSEMA CUNICULI* IN THE CELLS OF THE YOSHIDA RAT ASCITES SARCOMA

By

MICHAEL PETRI and TORBEN SCHIØDT

Received 15 ix 65

Some years ago a strain of a transplantable, malignant tumour, the Yoshida rat ascites sarcoma, displayed certain changes in the usual behavioural pattern It was noticed that although the malignant pattern was maintained, the survival time of the rats increased to some extent and the previously low survival rate rose from 2–3 per cent to about 20 per cent Surviving rats developed resistance towards sarcoma cells

When smears of the tumour were examined large numbers of a protozoan parasite were revealed in the sarcoma cells The parasite was shown to possess a filament (*Petri* 1965) and the experimental demonstration of filament extrusion classified the parasite within the *Microsporidia* This group of parasites is previously known only from lower animals, especially coelenterates and insects, but several recent reports have proved the occurrence of microsporidian parasites in various rodents (*Nelson* 1962, *Doby et al* 1963)

The parasite encountered in the sarcoma cells closely resembled *Encephalitozoon cuniculi*, so named by *Levaditi et al* (1923) and known to cause a silent infection in laboratory animals (*Perrin* 1943, *Jirovec* 1961) Identity could be assumed, however, when this parasite by *Weiser* (1964) was proved to be a microsporidian and also by *Lainson et al* (1964) These authors changed the name to *Nosema cuniculi* Other genera have not with certainty been established although *Weiser* (1965) proposed the name of *Nosema muris* to a microsporidian parasite found in white mice However, sufficient evidence from comparisons between the Nosemas described from mice have not yet been accumulated, and until then the parasite described in this paper is identified as *Nosema cuniculi* (see *Petri* 1966)

The parasitized sarcoma has been transplanted serially in rats in over 200 transfers for about three years Interesting and in some ways peculiar changes have been observed in the tumour host relationship as compared to the behaviour of a non infected sarcoma Pathogenicity towards mice has been observed (*Petri* 1966)

In Giemsa stained smears of the sarcoma cells the parasites are most often found in one or several vacuoles in the cytoplasm containing from 2 to about 60 parasites each The majority are mature spores but other forms are found presumably stages in a developmental cycle Great numbers of parasites can be harvested by aspirating ascites fluid as 10 20 per cent of the sarcoma cells contain parasites and prolonged search for parasites suitable for electron microscopy is not necessary Thus the difficulties which arise when the number of parasites is very low as in the ascitic fluid of Nosema infected mice (*Lainson et al* 1964) are not encountered

MATERIAL AND METHODS

One ml of ascitic fluid is aspirated from the peritoneum and injected immediately into 6 per cent glutar aldehyde The fluid is centrifuged for 5 minutes at 1000 r p m The sediment is taken through 3 changes of phosphate buffer (pH 6 8) Postfixation in 2 per cent OsO_4 centrifugation for 2 minutes at 1000 r p m

Embedding in Vestopal In the monomere phase the capsule was centrifuged for 5 minutes at 5000 r p m

Staining by 5 per cent equeous uranyl acetate or by lead hydroxyde after Reynolds Semifine sections are stained by toluidine blue

Ultrathin sections on the LKB ultratom I Examination in Siemens Elmiskop I

RESULTS

The parasites are located in vacuoles limited by membranes in the cyto plasm of the sarcoma cells The vacuolar space between the parasites is occupied by an electron lucid substance (Fig 1)

The length of the parasites is found to be in the vicinity of 2 microns

Outer membranes The surface of the parasite is formed by a cor rugated uneven membrane which limits an even electron lucid zone This zone is in some cases wider and less even presumably because the Vestopal in the monomere phase has not properly permeated the para site (Figs 3 a 4 a)

The innermost part of the surrounding membranes is formed by a delicate double membrane (Figs 2 3 4 a)

Inner membranes The larger part of the anterior end of the spore is occupied by a system of thin roughly parallel membranes which together constitute a laminated body (Figs 2 3 a) It seems reasonable to believe that it represents the polaroplast a term applied by *Huger* (1960) to a laminated structure in the spores of *Nosema locustae* The laminae are closely packed although in one spore only in groups of three (Fig 2) The whole body somewhate resembles the chloroplast

As in the work of *Huger* (1960) and of *Kudo & Daniels* (1963) the anterior part of the polar filament can be observed in the centre of the polaroplast (Figs 2 3 a)

A flattened sac like structure limited by a thin membrane was seen close to the anterior pole of the spore *i e* the pole at which the base of the polar filament is attached and seemingly modelled after the

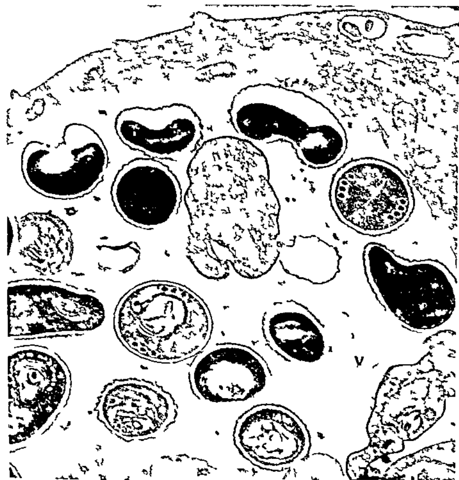


Fig 1

Survey of part of sarcoma cell with large vacuole (V) which contains several parasites They are seen in cross sections as well as in longitudinal sections (× 12000)

anterior half of the polaroplast circumference The interior of its widened central part in one section seems to continue into the polar filament (Figs 2 3 a)

The name *polar sac* is proposed for this structure which has not previously been described

A delicately membranous or filamentous substance is interposed between the polaroplast and the polar sac (Fig 2 middle left Fig 3 a) It may be vacuolated (Figs 2 lower left)

Polar filament In the posterior half of the spore not more than 5 cross sections of the filament can be seen in the periphery in each side of the parasite *Presumably* the filament curves in *oblique spirals* Surrounded by a clear halo which in earlier stages may be vesicular



F g 2

I art of Fig 1 at h gher magnification (× ?3000) Longitud nal section of parasite (1) sh ws the polar plast (I) and polar sac (arr w) The laminae of the p lar plast in parasite 2 are seen in groups of three Para tes 3 and 4 prob al l l d velopmental stages sh w a vacu le presumably the posteri r vacu l This vacu le can be seen in the m re matu re spore in the part f parasite 1 opp site the p laroplast Cross sect ns of the polar filament are surr nded by small ves cles (rro) in parasite 3

Fig 2) it has a dark shell and a dark core It measures approximately 0 1 micron Some of its coils are in contact with the polaroplast (Figs 2 3 a 4 a)

Granular substance A coarsely granular substance resembling rib o somes fills up a diffuse part of the spore (Figs 2 3 b 4 a) Another more delicately granular substance is distinctly delimited and presents a nucle ar like structure which is found in the middle or posterior part of the parasite

Vacuoles In the posterior half of some spores a larger or small r vacuole is found limited in part by irregular lamellae (Figs 2 3 b 4 It may contain a system of uneven membranes In one case coarse pr ecipitates are related to intravacuolar structures (Fig 2) They may be artefacts yet are not seen elsewhere in this figure

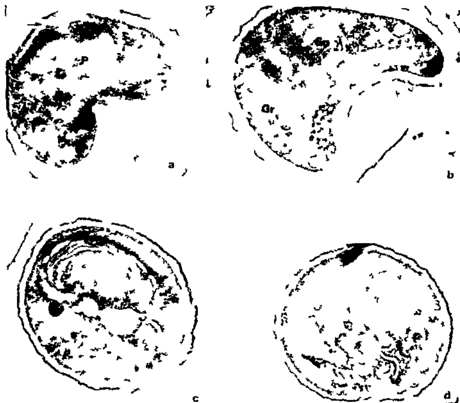


Fig 3

Fig 3 a shows the polar sac (arrow) in a T shaped section extending into the interior of the polaroplast (P) presumably as the beginning of the polar filament Fig 3 b shows the resemblance of the granular substance (Gr) to ribosomes and it shows the nuclear like structure probably the sporoplast (Sp) This picture also reveals the structure of the polar filament in five cross sections in each side of the parasite The vacuole of the parasite is seen in b c and d (× 26000)

Cytoplasmic organelles not found in the parasites In the present series of exposures neither mitochondria nor Golgi complex have been found Further ergastoplasm (rough membranes) has not been demonstrated

The host (sarcoma) cell Besides features common to malignant cells such as irregular nuclear outline and large nucleolus the following more special features of the host sarcoma cells have been noticed the nuclear membrane is considerably more indented on the side which faces the parasitic vacuole (Fig 5) And it is remarkable that the mitochondria all seem to be gathered between the vacuole and the nucleus

The nuclear membranes of the sarcoma cells are characterized by an extraordinary number of closely grouped nuclear pores with caps In tangential sections the distance between many of these caps and the nuclear membrane is remarkably large (Fig 6) There is further a

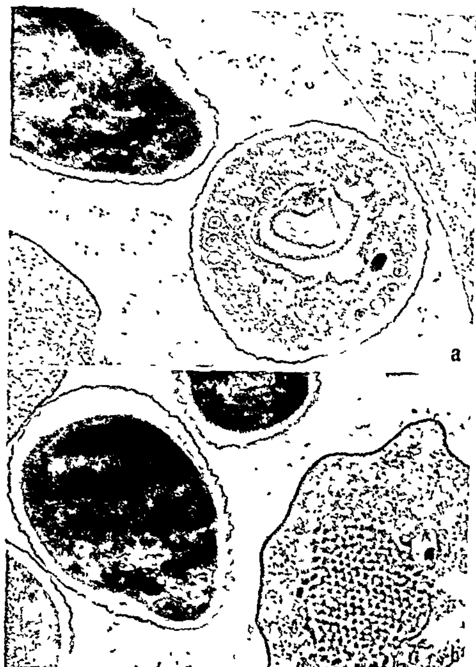


Fig 4

4 a In the upper left parasite the anterior part of the polar filament occupies the centre of the polaroplast The outer membranes are seen in the parasite to the right
4 b A cross section of a cytoplasmic bud from the sarcoma cell is probably seen to the right, limited by a thick membrane Inside, a honey comb like structure is seen (× 34000)



Fig 5

The relation between parasites and sarcoma cell The nucleus (N) is indented opposite the vacuole with the parasites and the mitochondriae (M) in the sarcoma cell cytoplasm are located between the nucleus and the vacuole (V) (× 11000)

large number of perichromatin granula ("Watson granula) in the sarcoma cell nucleus (Fig 6)

The large parasitic vacuole contains parasites as well as other bodies presumably parts of the host cell cytoplasm (Fig 1) It is noticeable that these bodies although closely resembling the sarcoma cell cytoplasm are limited by an external membrane which is considerably thicker than the plasma membrane as well as the vacuole membrane (Figs 1 4 a and 4 b) In one such body a peculiar, honeycomb like structure is present possibly a crystal (Fig 4 b)

While most of the constituents of the vacuole can be rather convincingly classified as either parasite or as part of the host cell a few



Fig 6

Sarcoma (host) cell nucleus Large accumulation of perichromatin granula (Pg) in the lower part of the nucleus A number of nuclear pore caps (arrows) are seen to to the left on the outer nuclear surface (× 33000)

structures cannot with certainty be put in one of these categories (e g Fig 2, top left) as the characteristic three-layered outer coating of the parasite body is lacking.

DISCUSSION

The two largest and most detailed studies on the ultrastructure of microsporidian spores have been published by *Huger* (1960) on *Nosema*

locustae and by *Kudo & Daniels* (1963) on *Thelohania californica* both parasitic in insects From these authors and from others (*Weiser* 1959 *Lom & Vavra* 1963 *Scholtysek & Danneel* 1962), a rough scheme of the ultrastructure of the microsporidian spore has appeared

The present work gives a preliminary sketch of the ultrastructure of *Nosema cuniculi* which in general fits quite well with this scheme It is the smallest microsporidian spore as yet studied by electron microscopy Indeed one cannot wonder that different species of organisms with a common function of high complexity that of sudden filament and sporoplasm extrusion possess a common structural pattern

As with the first named authors the polar filament runs backwards from the anterior pole through the centre of polaroplast Next it is seen in the periphery of the posterior half of the spore obviously in spiral coils These spiral coils can be seen in cross sections in the few electron micrographs from the work of *Lainson et al* (1964) which furnish few additional details The structure found by us is similar to that found by *Kudo & Daniels* although we have found no division into anterior and posterior coils

If the results of *Lom & Vavra* (1961) can be applied to all microsporidia the polar filament is tubular and the sporoplasm is released by passing through the interior of the extruded filament Although the filament in the present case does not seem hollow because of its electron dense centre it is not necessarily compact in the sense of not allowing the passage of the sporoplasm

It seems that the flattened structure which we have named the polar sac may in some way be involved in filament extrusion because of the close relation of its covering to the inner layer of the outer membrane and to the polar filament But further studies are needed to elucidate this question

The outer membranes are similar to the membranes found by *Kudo & Daniels* (1963) and by *Huger* (1960) although Huger s terminology is different

The size and exact extension of the sporoplasm the germ of the spore have not been finally settled by our material We believe that because of its nucleus like structure the more delicately granular substance which appears as a rather well limited body behind the polaroplast represents the sporoplasm or part of it

The coarsely granular substance is rather abundant It fills up the spore and surrounds the better defined structures

The polaroplast is thought (*Huger* 1960) to act as a swelling substance which may thus confer increasing hydrodynamic pressure upon the spore contents and thereby extrude or perhaps rather evaginate the filament and expel the sporoplasm There is however as yet no experimental proof of this The structure assumed to be the polaroplast by us in *Nosema cuniculi* differs in some respects from that of *Nosema locustae* (*Huger* 1960) and of *Thelohania californica* (*Kudo &

Daniels 1963) as a similary well-defined, laminated structure was not demonstrated by these authors, although Huger found lamination at higher magnifications. The polaroplast of *Thelohania californica* extends into the posterior part of the spore and displays a more variable structure, though some lamination is clearly shown. It is evident, however, that these laminae are much further apart that in *Nosema cuniculi* so that the polaroplast gets a more reticulated appearance.

The present material does not furnish any possibility to interpret the significance of the posterior vacuole. Studies of the ultrastructure of developmental stages are needed to throw light upon this structure.

SUMMARY

A study has been made of the ultrastructure of *Nosema cuniculi*, formerly known as *Encephalitozoon cuniculi*, in the cells of the Yoshida rat ascites sarcoma. Enveloped by a set of membranes the interior of the spore, which is about 2 microns long, contains a spirally coiled polar filament, traversing through a well-defined laminated structure assumed to be the polaroplast. A sporoplasm was present, but without differentiated structure. The polaroplast and the polar filament are closely related to a polar sac. Some special features of the host sarcoma cell are described.

REFERENCES

Doby J.-M., Alain Jeanne & Bernard Rault: Thelohania apodemi n. sp., première microsporidie du genre *Thelohania* observée chez un Mammifère. C. R. Acad. Sci. (Paris) *257*, 248 251, 1963.

Huger A.: Electron microscope study on the cytology of a microsporidian spore by means of ultra thin sectioning. J. Insect. Path. 2, 84 105, 1960.

Jirovec O.: Personal communication. 1961.

Kudo R. R. & Daniels E. W.: An electron microscope study of the spore of a microsporidian *Thelohania californica*. J. Protozool. *10*, 112 120, 1963.

Lainson R., Garnham P. C. C., Killick Kendrick, R. & Bird R. G.: Nosematosis, a microsporidial infection of rodents and other animals, including man. Brit. Med. J. *II*, 470 472, 1964.

Levaditi C., Nicolau S. & Schoen R.: L'etiologie de l'encephalite. C. R. Acad. Sci. (Paris) *177*, 985 988, 1923.

Lom J. & Vavra J.: Fine morphology of the spore in Microsporidia. Acta Protozool. *1*, 279 284, 1963.

Nelson J. B.: An intracellular parasite resembling a microsporidian associated with ascites in Swiss mice. Proc. Soc. Exp. Biol. Med. *109*, 714 717, 1962.

Perrin T. L.: Spontaneous and experimental Encephalitozoon infection in laboratory animals. Arch. Path. *36*, 559 567, 1943.

Petri M.: A cytolytic parasite in the cells of transplantable malignant tumours. Nature 205, 302, 1965.

Petri M.: The occurrence of *Nosema cuniculi* in the cells of transplantable malignant ascites tumours and its effect upon tumour and host. Acta path. et microbiol. scandinav. *66*, 13 30, 1966.

Schollyseck E. & Danneel Rolf: Über die Feinstruktur der Spore von Nosema apis. Deutsch. Entomol. Zeitschr. *9*, 471, 1962.

Weiser J.: On the taxonomic position of the genus *Encephalitozoon* Levaditi, Nicolau & Schoen 1923 (Protozoa, *Microsporidia*). Parasitology 54, 749 751, 1964.

Weiser J.: *Nosema muris* n. sp., a new microsporidian parasite of the white mouse ((Mus musculus L.) J. Protozool. *12*, 78 83, 1965.

Acta path et microbiol scandinav 66 447–464 1966

Universitetet i Oslo Institutt for Patologisk Anatomi
(Head Professor Olav Torgersen M D), Rikshospitalet, Oslo Norway

DEVELOPMENT OF MESOTHELIUM ON THE OUTER SURFACE OF INTRAPERITONEAL DIFFUSION CHAMBERS

By

GUNNAR ESKELAND and TROND ESKELAND

Received 27 ix 65

Diffusion chambers, constructed from filter membranes acting as a barrier to cells but not to fluids, have proved to be extremely versatile tools for *in vivo* cell cultures since they were introduced for this purpose in 1954 (1) Following intraperitoneal implantation, chambers made from filter membranes with a pore size less than 0 65 μ usually remain free from adhesions, provided they are sterile (8) After 7 10 days the free chambers are completely enclosed in a fibrous capsule (5, 20)

The life of cells within the chambers is entirely dependent on the passage of nutrients through this avascular tissue capsule as well as through the filter membrane Although the interference with diffusion must be considerable, both normal and malignant cells have been found viable within such chambers after 8–10 months (12, 14, 19)

It has previously been stated that plastic discs, placed in the peritoneal cavity of dogs (13) or in the scrotal cavity of rats (10) may remain non adherent and become covered by mesothelium

If free lying peritoneal implants are regularly covered by meso thelium, this would strongly support the hypothesis that the formation of a new mesothelial lining does not depend an on ingrowth in conti nuity from adjacent serosal cells

As a result of these considerations, we decided to study the tissue developing on the outside of intraperitoneal diffusion chambers

MATERIAL AND METHODS

Eighteen male rats 4–6 months old and weighing 200–300 g were operated on under anaesthesia with ether/alcohol (2 1) One diffusion chamber was implanted intra peritoneally in each animal through a midline incision which was then closed in two layers

The diffusion chambers were constructed primarily for other purposes and will

This investigation was supported by grants from *Doktor Alexander Malthes Legat* and from the *Norwegian Cancer Society (Landsforeningen mot Kreft)*

Daniels 1963) as a similary well-defined, laminated structure was not demonstrated by these authors, although Huger found lamination at higher magnifications The polaroplast of *Thelohania californica* extends into the posterior part of the spore and displays a more variable structure, though some lamination is clearly shown It is evident, however, that these laminae are much further apart that in *Nosema cuniculi* so that the polaroplast gets a more reticulated appearance

The present material does not furnish any possibility to interpret the significance of the posterior vacuole Studies of the ultrastructure of developmental stages are needed to throw light upon this structure

SUMMARY

A study has been made of the ultrastructure of *Nosema cuniculi*, formerly known as *Encephalitozoon cuniculi*, in the cells of the Yoshida rat ascites sarcoma Enveloped by a set of membranes the interior of the spore, which is about 2 microns long, contains a spirally coiled polar filament, traversing through a well-defined laminated structure assumed to be the polaroplast A sporoplasm was present, but without differentiated structure The polaroplast and the polar filament are closely related to a polar sac Some special features of the host sarcoma cell are described

REFERENCES

Doby J M Alain Jeanne & Bernard Rault Thelohania apodemi n sp première microsporidie du genre Thelohania observée chez un Mammifère C R Acad. Sci (Paris) 257 248 251 1963

Huger A Electron microscope study on the cytology of a microsporidian spore by means of ultra thin sectioning J Insect Path 2 84 105, 1960

Jirovec O Personal communication 1961

Kudo R R & Daniels E W An electron microscope study of the spore of a microsporidian *Thelohania californica* J Protozool 10 112 120 1963

Lainson R Garnham P C C Killick Kendrick R & Bird R G Nosematosis a microsporidial infection of rodents and other animals, including man Brit Med J II 470 472 1964

Levaditi C Nicolau S & Schoen R L'étiologie de l'encéphalite C R Acad Sci (Paris) 177 985-988 1923

Lom J & Vavra J Fine morphology of the spore in Microsporidia Acta Protozool 1 279 284 1963

Nature 205 302 1965

malig

micro

apis

Weiser J

& Sch

Weiser J

(Mus

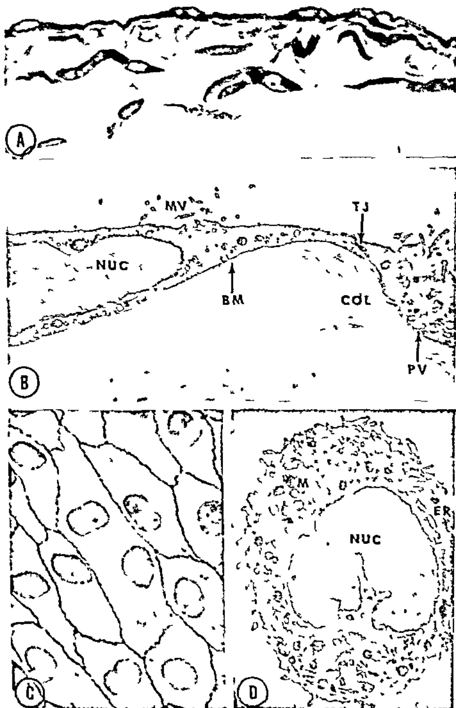


Fig 1

definite tissue could be seen with the naked eye when the chambers had been in place for 6 days or less

Light and electron microscopy revealed that every filter membrane was completely covered by cells By 2 days the vast majority of cells were of the same appearance as the peritoneal macrophages (Fig 2) They were arranged in one more seldom in two or three layers Occasional mast cells eosinophils and polymorphonuclear leucocytes were seen among the macrophage-type cells The macrophages sent long cytoplasmic processes into the pores of the filter membrane

In the later stages the number of cells and the thickness of the tissue increased and at the same time a difference between the cells of the deeper layers and the surface cells became evident For the sake of simplicity they will be described separately

The cells of the deeper layers By day 4 most cells were still of macrophage appearance With increasing periods of implantation a change in cell morphology was observed The cells nearest to the filter membrane were closely packed and usually retained their rounded shape and macrophage like appearance Superficial to these and increasing in number from the 4th day long and slender cells with tapering cytoplasmic processes were seen (Fig 4A Fig 6A and B) These cells were as a rule separated from each other by some intercellular substance Occasionally the plasma membranes of two cells were in apposition but intercellular contacts of the type tight junctions (11) were never observed The endoplasmic reticulum was well developed (Fig 7B Fig 8A) and the general appearance of these cells suggested that they were fibroblasts

Between the cells structures resembling collagen fibrils were seen on the 4th day Cross banding with a periodicity of approximately 640 Å was demonstrable in such fibrils on the 6th day (Fig 8B) The number of collagen fibrils increased with time (Fig 8D)

The surface cell layer On day 4 scattered cells that were large and of a flattened form were seen resting on the underlying cells (Fig 3A) In whole mount preparations a silver line pattern was sometimes observed reminding of the network of silver lines seen in mesothelium but incomplete and not so regular (Fig 3B)

Mitotic figures were frequently seen in the surface cells from day 4 onwards (Fig 6B)

Fig 2

Filter membrane from diffusion chamber 2 days in peritoneal cavity

A Light micrograph Macrophage type cells cover the surface and send cytoplasm processes into the pores of the filter membrane (HE) × 800

B Whole mount preparation of filter membrane × 800

C *Electron micrograph The cells covering the surface* of the membrane are of the same appearance as the peritoneal macrophages (compare with Fig 1D) ER — Endoplasmic reticulum G — Golgi zone M — Mitochondria × 1[illegible] 000

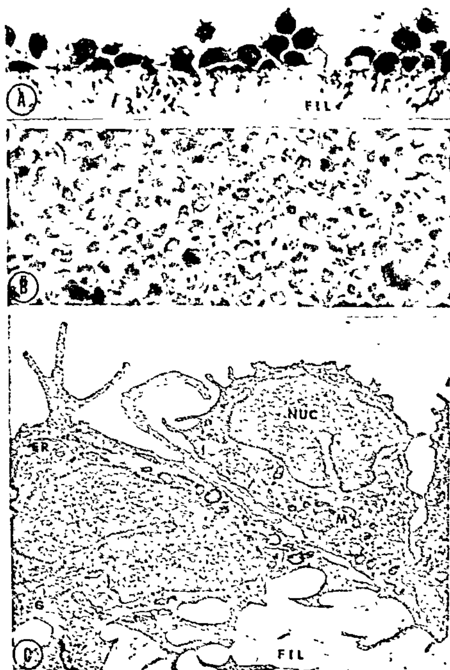


Fig 2

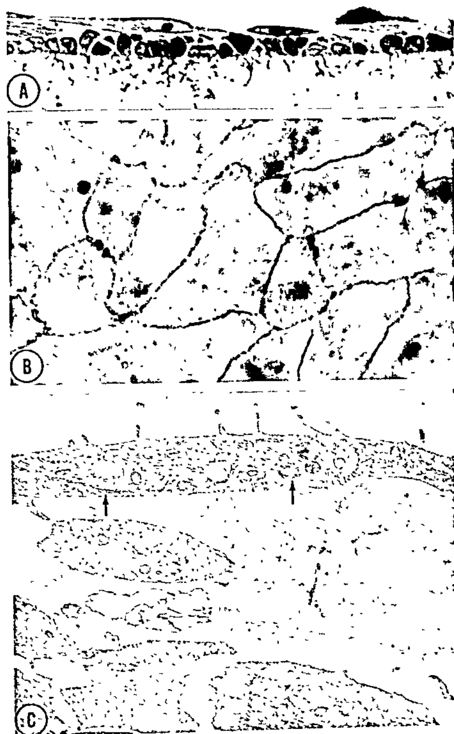


Fig. 3

Studied with the electron microscope, the flattened cells resembled fibroblasts, except that some of them displayed scattered cytoplasmic processes on the free surface (Fig 3C) These processes were slender and of a more uniform diameter than the irregular projections from the surface of macrophages, and resembled the microvilli of mesothelial cells "Tight junctions" were not observed at this stage, even if, as sometimes happened, the plasma membranes of two cells were in close apposition Neither was a basement membrane observed this early

In the course of 6 days, the majority of cells on the surface were large flat cells forming a continuous layer (Fig 4 A) In whole mount preparations a complete mosaic pattern of intercellular silver lines was found (Fig 4 B), very similar to that seen in Häutchen preparations of normal mesothelium (Fig 1 C) The lines of contact between neighbouring cells ran a varying course, some cells showed a more or less complex interdigitation (Fig 7 B), while others were apposed end to end (Fig 8 A) The cytoplasm of adjoining cells sometimes seemed to enclose a small space with microvilli projecting into it (Fig 7 A) Cell contacts of the type "tight junctions" were frequently observed close to the peritoneal surface (Fig 4 C Fig 7 B) Microvilli were projecting from the free surface of the cells (Fig 4 C), and sometimes a distinct, although incomplete, basement membrane was demonstrable (Figs 8 A and C)

These changes in the appearance of the cells seemed to occur simultaneously over the whole surface However, variations in the size and shape of the cells were frequently observed, especially with implantation periods up to 10 days The majority of cells were convincingly like mesothelial cells, while others were smaller and more rounded (Fig 5, Fig 6 B) Even these cells were in close apposition, and they had cytoplasmic processes similar to the microvilli of mesothelial cells (Fig 5 B)

On days 14 and 21, a complete layer of mesothelial like cells was found on the surface Some of these cells showed an abundance of microvilli (Fig 9 A and B)

The microvilli were up to 3 μ long and of a fairly uniform diameter 800–1200 Å At higher magnification fine filamentous structures were

---

Fig 3

Filter membrane 4 days in peritoneal cavity

A Light micrograph showing scattered flattened cells on the surface $\times$ 800

B Whole mount preparation Incomplete silver lines resembling those seen in mesothelium are present in some areas at this stage $\times$ 800

C Electron micrograph showing a flattened surface cell of the same type as those seen in Fig 1 A Note the slender cytoplasmic processes on the surface and the prominent endoplasmic reticulum (arrows) Fibrils are discernible in the intercellular space but characteristic cross banding was not demonstrable at this stage $\times$ 12 000

discernible within the microvilli; in cross section the filaments seemed to be arranged in a circular pattern (Fig 9C and D)

The cells constituting the surface layer possessed the main characteristics of mesothelium, as described in several studies (2, 9, 15, 21) They differed from normal mesothelial cells chiefly in two respects: They usually had a prominent endoplasmic reticulum and relatively few cytoplasmic vesicles, whereas normal mesothelial cells have a sparsely developed endoplasmic reticulum and numerous pinocytotic vesicles in their cytoplasm The significance of these differences will be discussed

DISCUSSION

The use of whole mount preparations of the filter membranes proved to be a valuable adjunct to the light and electron microscopic sections, since they permitted a survey of the whole surface which could hardly have been obtained by studying sections alone The demonstration, by treatment with silver nitrate solution, of intracellular border lines can only be achieved by methods of preparation permitting the inspection of the cells *en face*

There can be little doubt that the cells observed on the surface of the chambers in the early stages are peritoneal macrophages, which are known to be present in large numbers in the peritoneal fluid of the rat Neither can there be any serious doubt that the fibroblasts observed after some days are derived from the macrophages There is no other likely available source for these cells, and it is well documented that macrophages of this type readily transform into fibroblasts both *in vivo* and *in vitro*

The presence of cells with the characteristics of mesothelium on the surface of diffusion chambers has not, to our knowledge, been recorded previously In this study a complete lining of flattened surface cells was found on every chamber left in place for 8 days or more Silver treated whole mount preparations showed a mosaic pattern of distinct dark lines very similar to that seen in mesothelium Electron microscopy revealed cellular contact zones of the same kind as those described between mesothelial cells Tight junctions were frequently

---

Fig 4

Filter membrane 6 days in peritoneal cavity

A Light micrograph showing the "fibrous capsule" outermost line) by a continuous layer of flattened cells × 800

B Whole mount preparation, microscope focused on the superficial cell layer Silver lines closely resembling those seen in normal mesothelium (compare with Fig 1C) Some of the nuclei belong to cells in a deeper layer × 800

C Electron micrograph showing parts of two surface cells with microvilli (MV) a prominent endoplasmic reticulum (ER) and a tight junction (TJ) in the contact zone between the cells The long cell extension in the lower part of the illustration probably belongs to a fibroblast (F) × 18 000

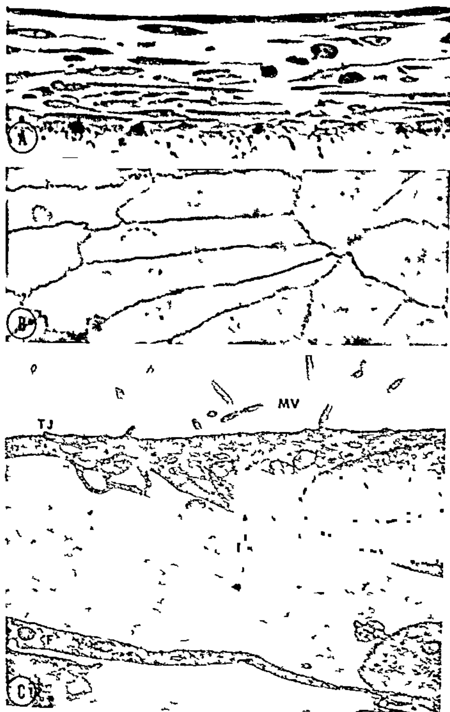


Fig 4

observed and a basement membrane was demonstrable from day 6. Microvilli were seen projecting from the cell surface identical in appearance to those described in normal mesothelium from several species.

Fine filamentous or possibly tubular structures similar to those observed in the present study have been demonstrated in the microvilli of pericardial mesothelium of cats and rats (21). Such filaments were not mentioned in a report on the ultrastructure of rabbit peritoneal mesothelium but this report was based on electron micrographs taken at low initial magnification and no information was given with regard to the methods used for increasing the contrast (2).

The surface cells in our study differ from normal mesothelium chiefly in two respects. They usually display a prominent endoplasmic reticulum and numerous ribosomes and they have relatively few cytoplasmic vesicles, especially in the early stages of implantation. In contrast, normal mesothelial cells have a sparsely developed endoplasmic reticulum and numerous pinocytotic vesicles.

It is commonly agreed that a prominent endoplasmic reticulum and numerous ribosomes reflect a high synthetic activity of the cell in question. Large numbers of ribosomes are usually found in relatively undifferentiated cells (16) and in endothelial cells a paucity of vesicles has been interpreted as a further sign of low differentiation (18). It is therefore not surprising that cells proliferating under the conditions offered to them in our experiments differ from normal mesothelium in these respects. Exactly the same variations from the normal picture have been observed in growing endothelium (4, 18, 22).

We conclude that the surface cells lining the outside of the diffusion chambers in our study are true mesothelial cells.

Since the chambers remain completely free from adhesions, this *implies that mesothelium may form without any direct connection with pre-existing mesothelium*. Secondly, it proves that mesothelial cells may live and proliferate on a completely avascular tissue. They must take their nutrition from the peritoneal fluid and they must permit a passage of fluid through or between the cells sufficient to keep the cells of the deeper layers alive.

The origin of these mesothelial cells is obscure. There are only two likely possibilities: 1) The free peritoneal macrophages may transform

---

Fig. 5

Filter membrane 8 days in peritoneal cavity.

A. Whole mount preparation showing variations in the appearance of surface cell. Macrophage-like cells to the left, while those to the right resemble normal mesothelial cells. × 800.

B. Electron micrograph. Surface cells of the same type as the cells to the left in Fig. 5A. The cells form a continuous layer and have long cytoplasmic processes resembling microvilli. × 12 000.

Fig 5





Fig. [illegible] (For text see page [illegible])



Fig 7 (For text see page 460)

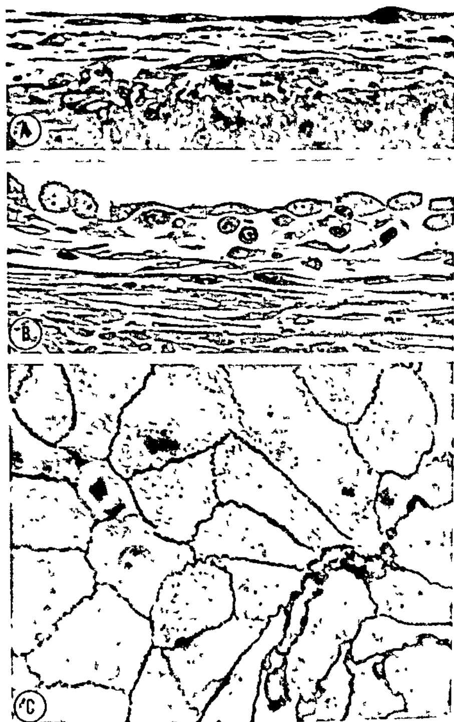
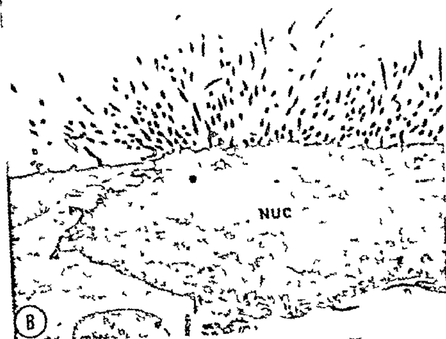


Fig. 6 (For text see page 460)



Fig 8





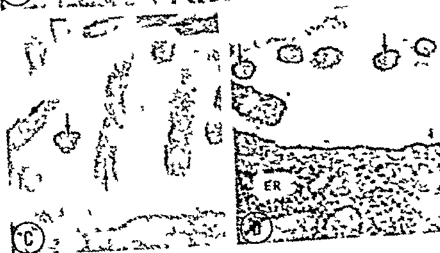


Fig. 3

revealed at the same time the presence of microvilli on the cell surface tight junctions between neighbouring cells, and a distinct, although not universally demonstrable basement membrane Cells were sometimes seen that might represent transitional forms between macrophages and mesothelial cells Superficially located cells, in close apposition and possessing microvilli but resembling macrophages in size and shape

2 If the new mesothelium had originated from grafted serosal cells one would expect to find isolated mesothelial cells or groups of cells as islets on the surface in the early stages and then an outgrowth of mesothelium from such islets To explain our observations according to the graft theory, one must stipulate either that these cells lose their mesothelial characteristics during the time they are being grafted or that a veritable shower of mesothelial grafts suddenly takes place between the 4th and the 6th day

However it is conceivable that the postulated detachment of mesothelial cells takes place only after a latency period or that detached mesothelial cells will not settle on the chamber until a suitable bed has been prepared for them by the peritoneal macrophages

Although the chambers are non adherent throughout the observation period there is obviously a close contact between parts of the chamber surface and the mesothelium lining the peritoneal cavity

Our findings do not disprove the theory of mesothelial grafting However, we believe that the morphological observations favour the other possibility *viz*, that the new mesothelium develops by the metaplasia of peritoneal mecrophages

SUMMARY

Diffusion chambers made from acrylic rings and Millipore HA filter membranes were implanted intraperitoneally in rats and left in place for 2–21 days Nearly all the chambers remained free in the peritoneal cavity Every free chamber was covered by cells which in the early stages were identical in appearance with the free peritoneal macrophages Fibroblasts were found later and typical collagen fibrils could be demonstrated from the 6th day after the implantation

On the surface of the tissue enclosing the chambers a complete lining of mesothelial cells was observed within 8 days

The possible origin of these mesothelial cells is discussed Our

---

Fig 9

Filter membrane 21 days in peritoneal cavity

A Light micrograph Some of the surface cells show an abundance of microvilli which are ordinarily not demonstrable in the light microscope × 800

B Electron micrograph of the cells marked with an arrow in Fig 9 A × 12 000

C and D Electron micrographs showing fine filamentous structures within the microvilli ER = endoplasmic reticulum × 60 000

observations suggest that new mesothelium may form by the metaplasia of peritoneal macrophages directly or *via* fibroblasts

REFERENCES

1 *Alg re G H Wea er J M & Prehn R T* Growth of cells *in v o* in diffusion chambers I Survival of homografts in immunized m ce J nat Cancer Inst 15 493 507 19 4

2 *Baradi A F & Hope J* Observations on ultrastructure of rabbit mesothelium Exp Cell Res 34 33-44 1964

3 *Cameron G R Hassan S M & De S N* Repair of Glisson s capsule after tangential wounds of the liver J Path Bact 73 1 10 1957

4 *Cliff W J* Observations on healing tissue A combined light and electron microscopic investigation Phil Trans B 246 305-325 1963

5 *Curran R C* The effect of dusts on L cells and peritoneal cells in diffusion chambers Ann N Y Acad Sci 86 916 928 1960

6 *Curran R C & Ager J A M* Tissue responses to filter membranes of various porosities Nature 185 1023 1024 1961

7 *Curran R C & Clark A E* Phagocytosis and fibre genesis in peritoneal implants in the rat J Path Bact 88 489 502 1964

8 *Curran R C & Rousell F* The application of the diffusion chamber technique to the study of silicosis J Path Bact 76 561 568 1958

9 *Eskeland G* Experimentelle Untersuchungen uber die Biologie des Peritoneums I Die morphologische Reaktion des Peritoneums auf Reize Det Norske Videnskaps Akademi i Oslo Oslo 1940

10 *Eskeland G* Regeneration of parietal peritoneum (Brief report) Acta path et microb ol scandinav 62 459–460 1964

11 *Farquhar M G & Palade G E* Junctional complexes in various epithelia J Cell B 1 17 375–412 1963

12 *Hays E F* Growth and morphology of mouse lymphoma cells in diffusion chambers Proc Soc exp Biol (N Y) 1 45 50 1961

13 *Howes F L* Personal communication to Brunschwig A & Robbins G F Regeneration of peritoneum Experimental observations and clinical experience in radical resections of intra abdominal cancer XV Congr Soc [illegible] 756 765

14 *Liel e* [illegible]

15 *Odor* [illegible] nd phase microscopes Amer J Anat 95 433 465 1954

16 *Palade G E* Microsomes and ribonucleoprotein particles In Microsomal Particles and Protein Synthesis Roberts R B Ed pp 36–61 Pergamon Press New York 1958

17 [illegible] lectron opaque stain

18 [illegible] generation of blood 1964

19 [illegible] Behaviour of three lymphomas in diffusion chambers in relation to their invasive capacity in the host J nat Cancer Inst 21 137 161 1958

20 *Shelton E & Rice M E* Growth of normal peritoneal cells in diffusion chambers A study in cell modulation Amer J Anat 105 281 341 1959

21 *Staubesand J & Schmidt W* Zur Histophysiologie des Herzbeutels I Mitteilung Elektronenmikroskopische Beobachtungen an den Deckzellen des Peri und Epikards Z Zellforsch 53 55–68 1960

22 *Warren B A & Brock L G* The electron microscopic features and fibrinolytic properties of "neo intima" Brit J exp Path 45 612 617 1964

Acta path et microbiol scandinav 66 465–470 1966

The Department of Pathology Malmö General Hospital, University of Lund Sweden

PLACENTAL CHORIOANGIOMATOSIS

By

TH BERGE

Received 2 x 65

Non malignant tumours of the placenta are rarely diagnosed The most common type is haemangioma also known as chorioangioma because it originates from chorial tissue Some investigators regard these new formations as true tumours, others, as malformations or hamartomas A case is described below in which the placentae in two consecutive pregnancies were sites of chorioangiomatosis with multiple, tumour-like structures in various stages of development

REPORT OF CASE

1963 The mother aged 17 was delivered of a macerated stillborn (1 760 g) at calculated term No hydramnion Necropsy revealed nothing remarkable

The placenta (450 g) showed the angiomatous structures of the same appearance as those described in the next section

1964 P[illegible] and delivery uncomplicated No hydramnion The child weighed [illegible]

invariably of firm elastic consistency The placenta also showed an infarction the size of a pigeon s egg

Microscopic findings Besides decidua of ordinary appearance for age and numerous regular chorionic villi several areas exhibited chorionic villi that had been converted into angioma like masses ranging in appearance from that of normal chorionic villi to that of large haemangiomas Some areas also displayed angioma developing in stem villi (Figs 3 4 5) Necrotic foci were observed in part of the tumour which was otherwise built up of stroma and blood vessels in varying proportions The stroma was very cellular throughout but in some areas it was oedematous and almost myxomatous The blood vessels which varied in calibre showed somewhat exuberant endothelium The surface of the lesions was covered by syncytial cells

DISCUSSION

As to the frequency of the tumour figures available vary widely Thus *Kuhnel* (1933) found 1 per 9 000 while *Marchetti* (1939), who traced as many as 200 cases in the literature found a frequency of 1 per 3 500 Thorough examinations of the placenta have, however shown that the tumour is much more common *Siddal* (1926), who fixed 600 placentae



Thumb-sized tumour extending to ma[illegible] rface.

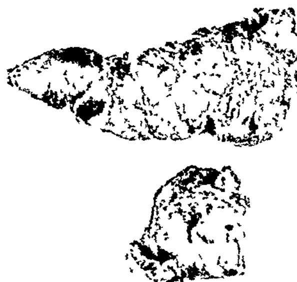


Fig 2
Placental tumours of varying size

and cut them in slices 5–8 mm thick, found 6 to harbour such tumours, i e 1 per 100 A similar frequency was reported by *Benirschke* (1962) *Wentworth* (1965) examined 620 placentae by using the Gough Wentworth large section technique and found tumours in 8 (1 per 77) of these, while *Shaw Dunn* (1959) in microscopical examination of sec-



Figs 3–4

Fig 3 Transitional forms between chorionic villi and chorioangioma (Haematoxylin eosin × 75)
Fig 4 Chorioangioma in stem villus (Haematoxylin eosin × 75)

tions placed 3 mm apart found tumours in 9 of 500 placentae examined (frequency 1 per 72) The frequency of the tumours does not vary with the age or parity of the mother (*Siddall* 1924, *Kuhnel* 1933)

The tumours vary widely in size, some being visible only under the microscope, others being as large as coconuts The tumours are usually solitary but occasionally multiple (*Beaufays* 1937, *Benirschke* 1962, *Kraus* 1903 *Rhamy* 1937) They are most often round or ovoid and have a smooth or nodular surface They vary in consistency and in appearance of the cut surface with the ratio between the amounts of vessels and stroma and with the extent of degenerative changes, if any



Fig 5
Part of a large tumour (Haematoxylin eosin × 115)

The tumour is often situated centrally on the foetal surface of the placenta Sometimes, however, they are localized to the periphery and or to the maternal side and occasionally, though rarely, to the umbilical cord (*Kuhnel* 1933)

Histologically, the tumour is built up of blood vessels and stroma, varying in relative amount from case to case as well as from one part of the growth to another. Oedematous or myxomatous degeneration of the stroma is common Some tumours have a superficial fibrous layer of varying thickness and often covered by a single layer of syncytial cells, while others, lacking the fibrous layer are covered only by syncytial cells Intact cells of Langhan's type are sometimes seen (*Shaw Dunn* 1959, *Dienst* 1903, *Rhamy* 1937, *Siddall* 1926)

Marchetti (1939) recognized three histological types, a vascular or mature type, a cellular or immature type, and a degenerative type often showing myxomatous degeneration of the stroma The vascular type is the most common one

Opinions differ widely as to whether the mass should be conceived as a true tumour or as a hamartoma (malformation) *Yule & O'Connor* (1964) think that it is a true tumour and that it arises from "a mature stem villus" *Meyer* (1923) feels that the tumour develops from stem villi that have failed to branch in a normal way to form functional villi The result is the formation of richly vascularised but non-functionating structures which he regards as a malformation, and when the mass assumes the character of a tumour it should be called a hamartoblastoma Most authors who have not seen transitional forms of the growth are inclined to regard it as a true tumour (*McInroy & Kelsey* 1954, *Kühnel* 1933, *Yule & O Connor* 1964) *Storch* (1878) described formations which he interpreted as transitional forms between chorio

nic villi and angioma And, judging from the case described by *Karnauchow* (1957) and that reported in this paper, true chorioangiomatosis with multiple tumours representing all transitional forms from angioma to normal villi, really do occur (Figs 3 4)

Neither has any unanimity been achieved regarding the clinical significance of the tumour One of the main points in the discussion is the possible relation between chorioangioma and hydramnion Some investigators claim that such a causal relationship does exist, stating that the frequency of co-existing hydramnion is 30 per cent (*Siddall* 1924, de *Costa et al* 1956, *Szathmary* 1934) According to *Marchetti* (1939), however, the frequency of hydramnion, abortion, and premature births *is not higher than the* frequency ascribable to chance *Resnick* (1953) also reported a correlation between oligohydramnion and chorioangioma

The case described above is of special interest because it shows that in chorioangiomatosis tumours develop from stem villi as well as from successively transformed peripheral villi The diffuse nature of the process in two consecutive pregnancies, the demonstration of various transitional forms between normal chorionic villi and fully developed angioma suggest that the condition should be regarded as a malformation possibly an anomaly of the anlage But is is not a malformation of the type described by *Meyer* (1923), for the angiomatous structures developed also from fully branched villi Inquiry into the familial history revealed no heredity for angioma Naturally, the possibility that the condition in our case might be a multifocal effect of a neoplasm inducing stimulus, cannot be excluded

SUMMARY

A report is given of placental chorioangiomatosis in two consecutive pregnancies, one terminating in delivery of a premature macerated foetus, the other in the uncomplicated delivery of a normal child Histological examination on both occasions showed diffuse chorioangiomatosis originating from stem villi and from peripheral villi and exhibiting a wide variety of transitional forms between normal villi and fully developed angioma

REFERENCES

Beaufays J Ein Hamartoblastom der Placenta Diffuses Angioblastom Arch F Gynak *164* 646–652 1937

Benirschke K A review of the pathologic anatomy of the human placenta Am J Obst & Gyn *84* 1595–1622 1962

de Costa E J Gerbie A B Andresen R H & Gallanis T C Placental Tumours Hemangiomas With special reference to an associated clinical syndrome Obst & Gyn 7 249 259 1956

Dienst A Ueber den Bau und die Histogenese der Placentargeschwulste Z schr f Geburtsh u Gynäk *48* 191 261 1903

Karnauchow P N Chorioangiomatosis of Placenta Report of a case Obst & Gyn *9* 317 321 1957



Fig 5
Part of a large tumour (Haematoxylin eosin × 115)

The tumour is often situated centrally on the foetal surface of the placenta Sometimes, however, they are localized to the periphery and/or to the maternal *side* and occasionally, though rarely, to the umbilical *cord* (*Kuhnel* 1933)

Histologically, the tumour is built up of blood vessels and stroma, varying in relative amount from case to case as well as from one part of the growth to another Oedematous or myxomatous degeneration of the stroma is common Some tumours have a superficial fibrous layer of varying thickness and often covered by a single layer of syncytial cells, while others, lacking the fibrous layer are covered only by syncytial cells Intact cells of Langhan's type are sometimes seen (*Shaw Dunn* 1959, *Dienst* 1903, *Rhamy* 1937, *Siddall* 1926)

Marchetti (1939) recognized three histological types a vascular or mature type, a cellular or immature type, and a degenerative type often showing myxomatous degeneration of the stroma The vascular type is the most common one

Opinions differ widely as to whether the mass should be conceived as a true tumour or as a hamartoma (*malformation*) *Yule & O'Connor* (1964) think that it is a true tumour and that it arises from "a mature stem villus" *Meyer* (1923) feels that the tumour develops from stem villi that have failed to branch in a normal way to form functional villi The result is the formation of richly vascularised but non-functionating structures which he regards as a malformation and when the mass assumes the character of a tumour it should be called a hamartoblastoma Most authors who have not seen transitional forms of the growth are inclined to regard it as a true tumour (*McInroy & Kelsey* 1954, *Kuhnel* 1933, *Yule & O'Connor* 1964) *Storch* (1878) described formations which he interpreted as transitional forms between chorio-

Ullevål Hospital, Deepartment of Pathology, Oslo, Norway
Head Professor Kristen Arnesen, MD

THE ANITSCHKOW MYOCYTE

Further Evidence of its Myogenic Origin and Non-Rheumatic Genesis

By

ARNE SERCK-HANSSEN

Received 25 viii 65

The Anitschkow myocyte is a cell found in the heart and distinguished by its nuclear structure, characterized by a centrally placed, longitudinal bar of chromatin with a serrated edge. This chromatin bar is separated from the nuclear membrane by a clear zone, except for occasional threadlike connections from the bar to the nuclear membrane These features are the basis for the commonly used descriptive names "caterpillar cell' when the cell is seen in longitudenal section, and "owl eyed cell" when seen in transverse section (Fig 1 a and b)

Cells with nuclear characteristics as above, were observed and first described superficially by von *Oppel* (1901) in a study on the reaction of the myocardium to foreign bodies, and by *Saltykow* (1905) It was Anitschkow, however, who in 1913 gave a full description of the cell that later has borne his name Anitschkows beautiful drawing clearly demonstrate his perception of the origin of the cell from the cardiac striated muscle cell He believed that the myocyte plays an important part in the formation of granulation tissue in the myocardium, and that it eventually ends up as a cell indistinguishable from the fibroblast

In 1929, *Wenezianowa-Grusdkowa* challenged the view of the myogenic origin of these cells, concluding that they probably represented circulating cells of the reticuloendothelial system

Jaffe (1938) concluded that the cell was a fixed histiocyte

Ehrlich & Lapan (1939) supported the view of the reticuloendothelial origin and proposed the name "myocardial histiocyte"

Clawson (1941) in a study of hearts with rheumatic stigmata concluded that the Anitschkow myocyte should be regarded as a cardiac histiocyte, and expressed the view that the cell exhibited a marked proliferative activity in rheumatic inflammation

Rubenstone & Saphir (1962) concluded "The Anitschkow cell is neither a myocyte nor myogenic"

After this long period, in which the myogenic origin of the cell was



Fig 1

Fig 1a 'Caterpillar cell Case 5 H & E × 2500
Fig 1b 'Owl eyed" cell Case 5 H & E × 2500

disputed, *Murphy* (1963) in a beautifully illustrated paper co
cingly demonstrated the myogenic origin of cells with characterist
the Anitschkow myocyte He further demonstrated that these
could originate not only from cardiac striated muscle cells, but
from smooth muscle cells found in the connective tissue betw
endocardium and myocardium and in the cardiac valves, as wel
from smooth muscle cells of the coronary vessels

He also concluded that the Aschoff body, beeing characteristic
active rheumatic heart disease, really was a focal lesion of muscle ce
some of which exhibited the characteristics of the Anitschkow myocy

The purpose of this paper is to present further evidence of th
myogenic origin of the Anitschkow myocyte and to demonstrate th
large number of cells with these features may be present in cases i
which generalized or myocardial hypoxia is a common factor, and in
which there are no signs of rheumatic inflammatory reaction

MATERIAL AND METHODS

le 1 presents short clinical and autopsy data of the presented cases all from
nts found in the course of routine histological examination
ses 1 2 and 3 were selected because they represent eo ipso a unique experi
tal model whereby the coronary arteries are supplied by a mixture of arterial
venous blood
es 4 and 5 were selected as representatives of a number of similar cases of
ne membrane disease or intra uterine asphyxia all revealing large number of
hkow cells in the myocardium
ks of tissue were taken from the intraventricular septum and the left ventri
vall All tissues were fixed in 4 per cent formalin passed through the standard
ating series of alcohols and embedded in paraffin Sections were stained with
toxylin and eosin one section from each block only was examined The
s in the myocardium only will be described and discussed

TABLE 1

	Case 1	Case 2	Case 3	Case 4	Case 5
Name and sex	J U ♂	J K ♂	W R ♂	N F M ♂	N A ♂
Autopsy No	810 64	825 64	1224 64	303 65	310,65
Weight at birth in grams	3585	1130	2900	1810	2350
Age at death	3 days	17 hrs	4½ days	10 hrs	Stillborn
Cause of death	Aortic atresia and mitral stenosis	Aortic atresia and mitral stenosis	Aortic atresia and mitral stenosis	Pulm atelect Aspiration Hyalin memb	Placental insuff Infarcts & Haemorrhage

defects of somewhat varying size

Case 4 was the second of twins delivered by forceps Cried only after resuscita t on. Was cyanotic all the time Developed increasing respiratory distress with chest resession before death

Case 5 was born by a mother who had proteinuria and hypertens on The foetal heart beat was heard until shortly before birth

OBSERVATIONS

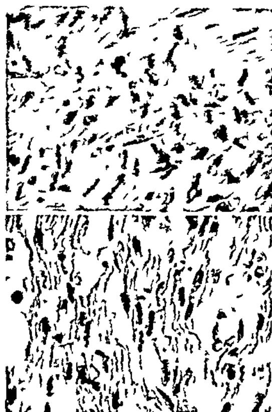
In all cases groups of striated muscle fibres with nuclei exhibiting the features of the Anitschkow myocyte were seen (Figs 1 2 3 4 5 6 7) In some areas naked nuclei with the same features were seen in the adjoining interstitial tissue (Figs 6 and7) No areas of necrosis or obvious fragmentation of the muscle fibres were seen nor any cellular infiltration suggesting an inflammatory reaction

DISCUSSION

The presented cases represent 3 different situations

1 Intrauterine and post natal myocardial hypoxia Cases 1 2 and 3
2 Neonatal generalized hypoxia Case 4
3 Intrauterine generalized hypoxia Case 5

In Cases1 2 and 3 a relative myocardial hypoxia must have persisted from the time of foetal life when partitioning of the truncus arteriosus took place The hypoxia must have been accentuated after birth when all the oxygenated blood from the lungs in order to leave the heart must have passed through the fairly small opening in the septum primum Pre and postnatally the coronary supply has been main tained by a reversed flow in the hypoplastic ascending aorta of mixed



Figs 2 3

Fig 2 Large number of Anitschkow cells Case 1 H & E × 550
Fig 3 Large number of Anitschkow cells Case 2 H & E × 550

arterial and venous blood reaching the systemic circulation through the ductus arteriousus

In Case 4 a generalized hypoxia was present from birth It is possible that some intrauterine hypoxia may have been present due to placental insufficiency, although there was no indication of this The hypoxia therefore probably did not last for more than 10 hrs

In Case 5 a general intrauterine hypoxia must have been present The placental infarcts were widespread apparently of long duration *and probably not the direct cause of the hypoxia The retroplacental* haematoma must be regarded as a complication of the mothers toxaemia The separation probably occurred 24 hrs prior to birth at the most

In the presented cases the common feature is hypoxia The duration of the hypoxia has varied from several months to probably no longer than 10 hrs

There can be little doubt that the nuclear structure of a large number of striated cardiac muscle fibres as presented in Figs 1 to 7 comply with the features of the Anitschkow cell It is also evident that



Figs 4–7

Fig 4 Nuclei in striated myocardial fibres with the features of the Anitschkow myocyte Case 3 H & E × 1300
Fig 5 Large number of Anitschkow cells Case 4 H & E × 500
Fig 6 Naked nuclei (arrows) and cell with small amount of cytoplasm in interstitial tissue (window) Case 5 H & E × 550
Fig 7 Greater magnification of window in Fig 6 showing cytoplasmatic remnants H & E × 2500

nuclei with the same features are seen with small remnants of cytoplasm (Fig 7) or as naked nuclei lying in interstitial tissue (Fig 6) This is in full accordance with the findings of previous workers cited above who accept the view that the nucleus with these features is of myogenic origin We share this view

We also agree with the statement by *Ehrlich & Lapan* (1939) that occasional naked nuclei may be found throughout the entire musculature in the average normal human heart preferably in the interfascicular planes It was our impression, however, that the cells were more numerous in patients who had stigmata of rheumatic infections Therefore the finding of a large number of these nuclei in the myocardium of infants in whom the only probable explanation was hypoxia, arose our interest We feel, however, that the cell probably is a manifestation of myocardial cell damage short of necrosis, and that the causes may be many, hypoxia being one of them

To test this hypothesis in relation to the presented cases it would be necessary to examine the hearts of infants in the appropriate age groups where death was not preceded by a period of hypoxia As most neonatal deaths are preceded by a period of hypoxia such a control material has not been available Amongst the "cot deaths", which probably would be the nearest to sudden deaths amongst previously healthy children, infants in the appropriate age groups are most unusual Amongst 5 cases of "cot deaths" aged between 3 months and 2½ years, myocardial changes of the nature described above were not noted although occasional cells complying with the features of the Anitschkow myocyte could be seen

Thus, with regard to this cell, our knowledge has not advanced, since Anitschkow 50 years ago stated "Der Hauptteil dieser Elemente ensteht infolge einer Degeneration der kontraktilen Muskelsubstanz, d h beim Zugrundegehen der Muskelfasern als differenzierte histologische Elemente

SUMMARY

A large number of cells with the characteristic features of the Anitschkow myocyte were found in the myocardium of 5 infants One child was stillborn, the others died at ages of from 10 hrs to 4½ days

The myogenic origin of these cells is demonstrated In the presented cases, the genesis of the cells is believed to be hypoxia In 3 cases the cause of the hypoxia was congenital heart disease, in 1 case pulmonary atelectasis with hyaline membranes and in 1 case placental insufficiency

REFERENCES

Anitschkow N Experimentelle Untersuchungen uber die Neubildung des Granulationsgewebes im Herzmuskel Zeitr z path Anat u z allg Path 55 373–415 1913

Clawson B J Relation of the Anitschkow myocyte to rheumatic inflammation Arch path 32 760–763 1941

Ehrlich J C & Lapan B The Anitschkow Myocyte Arch path 28 361–370 1939

Jaffe R H The reticulo endothelial system in Downey H Handbook of hematology, New York, Paul B Hoeber Inc vol 2 pp 1008 and 1089 1938

Murphy G E The characteristic rheumatic lesions of striated and non striated or smooth muscle cells of the heart Medicine 42 73 118 1963

Rubenstone A I & Saphir O Myocardial reactions to induced necrosis and foreign bodies with particular reference to the role of the Anitschkow myocyte Lab Invest *11* 791–807 1962
Saltykow S Uber diffuse Myocarditis Virchows Arch. f path Anat *182* 1 39 1905
von Oppel W Uber Veranderungen des Myocardes unter der Einwirkung von Fremdkorpern Virchows Arch f path Anat *164* 406–436 1901
Wene ianowa Grusdkowa M S Zur Frage des Ursprunges der sogenannten Myozyten im Myokardium Frankfurt Ztschr f Path 37 538–549 1929

Acta path et microbiol scandinav 66 478 484 1966

Department of Pathology, Bispebjerg Hospital Copenhagen Denmark
Head Charles Johansen M D

AN UNUSUAL CASE OF MALIGNANT PANCREATIC CYST

By

AAGE JOHANSEN

Received 27 x 65

The rareness with which abdominal cysts are encountered is one of the principal causes for the incompleteness of the data dealing with them Except for ovarian cysts our knowledge concerning this subject is based mainly on surgical case reports, which only make brief mention of the pathology of the cysts

Abdominal cysts may be found in intimate relation to any fully developed organ, or they can be found in the retroperitoneum, the mesentery or the omentum

The connection with certain organs may facilitate the diagnosis Besides the ovarian cysts, cysts and cystic tumours of rather well known nature take their origin from the spleen, the pancreas the liver, and the kidney and more rarely from other abdominal organs

The following case illustrates the contrary, since the relation of the cyst to its neighbouring organ seemed to impede the diagnosis

CASE REPORT

The patient was a woman aged 69 Her past history was negative with the exception of a strumectomy 20 years earlier for Grave s disease

A month prior to admission she developed a pain in the upper left quadrant of the abdomen radiating to the back Though she had lost two kg in weight she had noticed a slight increase of the abdomen

Examinations disclosed a mass extending from the ribs on the left side down into the pelvis First the tumour was thought to be an enlarged spleen but blood examinations were negative A diagnostic "splenic" puncture showed that the tumour was cystic The contents were brown Cytological examination revealed many erythrocytes leucocytes and a few cells of uncertain nature apparently not tumour cells X ray examinations showed a soft mass in the left hypochondrium with calcification in the periphery The left kidney and the transverse colon were dislocated downwards while the stomach and the oesophagus were displaced to the right

Laboratory Results at Admission

Haemoglobin 11 4 g per cent Red corpuscles 3 700 000 Leucocytes 5 700 Thrombocytes 470 000 Differential count Polymorphonuclear neutrophils 64 per cent eosinophils 1 per cent lymphocytes 30 per cent monocytes 5 per cent The fragility of the red cells was normal with a bleeding time of two min Urine examination was negative Serumcreatinine 0 7 mg% serumchlorid 103 meq/l serumsodium 23 1



Fig 1

A and B Part of the cystwall near to the spleen Macrophages and cholesterol crystals are seen (Spleenic tissue downward (A) and to the left (B)) (× 60) C Pancreatic islets in the cystwall (× 80) (H & E)

mM/l serum potassium 4 6 meq/l serumprotein 7 0 per cent with a slight elevation of the α_2 albumin fraction Sedimentation rate was 81 mm/hour

An explorative laporatomy was performed (Chief surgeon K H Koester) There was no ascites and no carcinosis A cystic tumour the size of a football or more was seen situated to the left of the stomach displacing the splenic flexure of the colon downwards The ligamentum gastro-colicum was stretched over the cyst There were massive adhesions to the neighbouring organs The spleen was incor

on the diaphragma and the stomach The adhesions to the pancreas were easily separated This organ was found to be quite normal

The postoperative course was uncomplicated and the patient left the hospital in good state

Two and a half years later she was readmitted with signs of metastases and ascites She spent one and a half month in the department had an apoplectic insult and died

Pathological Examination

The specimen consisted of a large cyst nearly spherical measuring 30 cm in diameter The cyst had been opened and was empty It was unilocular The wall which was mainly fibrous ranged in thickness from 3 mm to a few cm In the

cystwall

Microscopically sections from different regions of the wall of the cyst from the spleen and especially from the region of transition were examined

The wall consisted mainly of connective tissue which was partly hyalinized containing only few cells though the interior part in some places near the spleen revealed a looser tissue in which many fat laden macrophages old bloodpigment

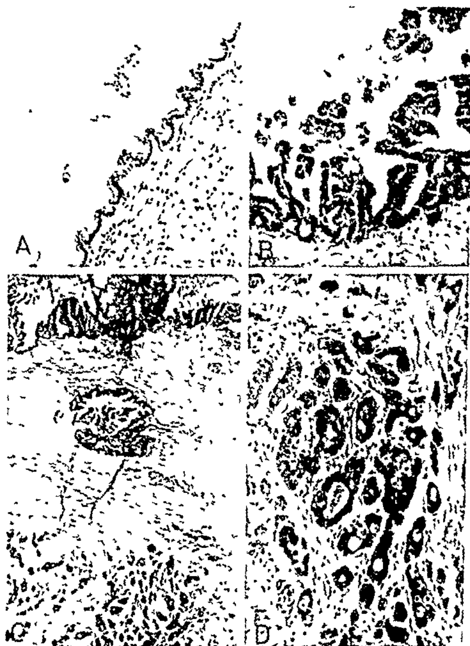


Fig 2

A The papillary lining of the cystwall (× 110) B Malignant transformation (× 115) C Malignant epithelial elements in the cystwall (× 60) D High power view of tumour tissue in C (× 400) (H & F)

and a great number of cholesterol crystals were seen (Fig 1A and B) In this region there were many newly formed thinwalled small bloodvessels

On the exterior of the cyst a layer of flattened cells was seen

The inner side of the cyst especially near the spleen was covered by a medium tall columnar epithelium which in many places was desquamated The epithelium was papillary (Fig 2A) The papillae were generally small, but in some places they were larger and ramified and some of them showed signs of malignancy such as *cellular deformities, nuclear dedifferentiation, and a few mitoses* (*Fig 2B and C*) Deeper in the cystwall epithelial elements forming sometimes greater but more frequently smaller and badly defined acini were seen (Fig 2D)

In one of the sections from the wall taken a few cm from the spleen a few normal or slightly hypertrophic pancreatic islets were seen (Fig 1C) No exocrine pancreatic tissue was seen in any section

The spleen itself showed a slight reticulosis and fibrosis and signs of inflammation with capillary formations Cholesterolcrystals and haemosiderin were also found. No sections were taken from the calcified part

Autopsy

The body was that of an elderly, emaciated woman There was no icterus and no oedema

The peritoneal cavity was opened It contained 1200 ml of a yellow serous fluid A tumour mass about 12 cm in diameter was seen in the splenic region The tumour was surrounded by fibrous tissue making a sort of capsule It was soft fragile and necrotic The peritoneal surface showed disseminated areas of yellowish white tumour tissue This was also seen on the surface of the liver, but there were no metastases to the parenchyma which showed a characteristic chronic congestion The gall bladder and the common duct were normal

There was no relation between the tumour masses and the pancreas This organ was of normal size The surface and the cut surface were quite normal There were no cysts anywhere The pancreatic duct was opened in its whole extent. There were no dilatations and no calculi

The stomach and the intestines disclosed no abnormalities of the mucosa

The kidney and the adrenals were without alterations

The ovaries were both small and fibrotic The Fallopian tubes and the uterus were normal corresponding to the age.

With the exception of many smaller pneumonic foci in both lungs and a slight arteriosclerosis in the aorta and the coronary arteries none of the other organs were [illegible]

a
s

DISCUSSION

The fact that the spleen was incorporated in the cystwall made it natural first to consider this organ as the original site of the cyst Splenic cysts are relatively wellknown and since the first report by *Andral* (1829) many hundred have been published (Danish cases *Trolle* 1943, *Zilstorff-Petersen* 1951, *Jensen* 1954) The majority are parasitic cysts, and *Fowler* (1913) was able to collect 191 cases from the literature before 1891 In 1924 he reported 90 cases of nonparasitic cysts Classifications of the last group have been made by *Lubarsch* (1927), *Pool & Stillman* (1923) and based on pathogenetic considerations by *Fowler* (1924) In accordance with the latter, splenic cysts may be 1 primary or true cysts, including dilatationcysts and true neoplastic cysts, *i e* lymphangiomas and haemangiomas or 2 se-

condary or false cysts including degenerative cysts, inflammatory cysts and traumatic cysts

The traumatic cysts, which according to many authors (*Brandberg* 1928, *Snyder & Rezek* 1943, *Pool & Stillman* 1923) are the most frequent nonparasitic splenic cysts, exhibit a structure which has much in common with the actual case They are usually big and unilocular, have a fibrous wall with signs of old haemorrhages and chylous effusion due to ruptured lymphatics In accordance with this their contents often are haemorrhagic There may be calcified areas in the wall However, traumatic cysts are without epithelial lining or at the most there is a single layer of flat cells growing in from the lymphatics in connection with the cavity, making the diagnosis against lymphangiomas difficult

In this cyst there was a distinct epithelial lining of the wall and in addition papillary excrescences In many parts the cyst had an appearence completely like an ovarian cystadenoma

Cases of splenic cysts with a similar ovarian like structure have been reported on two occasions in the literature, (*Brandberg* 1928, *Schultze Heubach* 1921) and both were interpreted as metastases from malig nant ovarian pseudomucinous cystadenomas operated 25 and 11 years previously In our case the ovaries were quite normal

The discovery of the undoubtedly pancreatic elements in the cyst wall increased the diagnostic possibilities but also the difficulties

During the operation only slight adhesions between the cyst and the pancreas were found and at autopsy the pancreas was normal If the cyst is considered as being of pancreatic origin it may have developed in ectopic pancreatic tissue or the growth may have been so expan sive, that the cyst—maybe in combination with a trauma—detached itself from the pancreas and included the spleen in its wall

Several cysts and cystic tumours take their origin in the pancreas The traumatic cysts, often known as pseudocysts or omentum minus cysts, are situated outside the pancreas on account of their genesis which is outpouring of digestive ferments into the lesser sac (*Richman* 1956) They often contain enzymes (*Meyer et al* 1949) Since there was no suspicion of this in the present case enzymeanalysis was not performed The microscopic appearence of the cysts is identical with that of traumatic splenic cysts and there is no epithelial lining

The possibility that the actual case could be a pancreatic retention cyst also had to be considered Such cysts usually have an epithelial lining which however may be desquamated in inflammatory con ditions Retentioncysts which like traumatic cysts are unilocular origi nally develop in the parenchyma but when growth is extensive they may loose their connection with this organ Unfortunately, the com pletely normal appearence of the pancreas had the effect that no microscopic examination of the organ was performed There were no calculi and no calcifications to explain a possible retention but a slight

pancreatitis or the basal cell metaplasia of *Korpassy* (1939) could not with certainty be excluded

The great tendency of the epithelium to form papillary proliferations followed by regular tumour formation is not in agreement with the genesis of retention but suggests a true neoplasm Next to pseudocysts cystadenomas are the most frequently encountered cystic tumours of the pancreas (*Mozan* 1951) Papillary formations especially in the larger ones are not uncommon (*Frant* 1959) and malignant changes have been described (*Sawyer* 1964 *Iversen & Johansen* 1965) Cystadenomas usually are multilocular they develop from the duct epithelium (*Glenner & Mallory* 1956) Probably as in the ovarian cystadenomas pressure atrophy of the septa may take place so that large tumours may be unilocular

The papillary portion of the cyst from our patient showed great similarity with malignant cystadenocarcinomas as we have seen them in this department In the common pancreatic adenocarcinomas the cylindric cell carcinoma of *Ewing* (1940) or the large duct carcinoma of Frantz (1959) there may be cyst formation with papillary excrescences but usually the cysts are small It has to be remembered that both the cystadenocarcinoma and the usual adenocarcinoma are of ductal origin and that acinar cells and islet cells both arise from duct epithelium

The small glandularlike elements in the cystwall have a slight resemblence to malignantly changed acinar cells or they may be small ducts having the same origin as the cyst The islet cells certainly persist from the pancreas since the high differentiation of the duct epithelium made it improbable that the islets should rise from these cells

Other aethiological possibilities have also been considered Mesotheliomas Wolffian cysts *Handfield Jones* (1924) mesocolic cysts renal cysts and others from this region have been reported The presence of pancreatic tissue in the wall of the cyst made this improbable

CONCLUSION

In spite of the intimate relation between the spleen and the cyst the latter had to be interpreted as being of pancreatic origin The epithelial lining of the cavity and the pancreatic islet tissue in the cystwall seem to justify such a contention The strongly expansive growth of the cyst may be responsible for the detachment from the pancreas and the taking up of the spleen into the wall Origin in ectopic pancreatic tissue is also a possibility

The size of the cyst and its unilocular structure are in good agreement with a pancreatic retention cyst but the pronounced tendency to proliferation of the epithelium is indicative of a real neoplasm probably a cystadenoma in which the cystic elements are more pronounced than the adenomatous

SUMMARY

An unusual case of cystadenocarcinoma pancreatis which appeared as a splenic cyst is reported The pathology of splenic and pancreatic cystic tumours are briefly mentioned

REFERENCES

Brandberg R Contribution to the knowledge of Non parasitic Cysts of the Spleen Acta Chir Scand 63 346 373 1928
Ewing J Neoplastic Diseases A Treatise on Tumours ed 4 W B Saunders Co Philadelphia pp 764–765 1940
Frantz V K Tumors of the Pancreas Atlas of Tumor Pathology Armed Forces Institute of Pathology Washington 1959
Fowler R H Cysts of the spleen A radiological and Surgical study Ann Surg 57 658 690 1913
Fowler R H Further study of cysts of the spleen Ann Surg 80 58 61 1924
Glenner G G & Mallory G K The cystadenoma and related nonfunctional tumors of the pancreas Pathogenesis classification and significance Cancer 9 980–996 1956
Handfield-Jones R M Retroperitoneal cysts Their pathology diagnosis and treatment Brit J Surg 12 119 134 1924
Henke F & Lubarsch O Handbuch der speziellen pathologischen Anatomi und Histologi Bind I 2 Julius Springer Berlin 1926
Meyer K A Sheridan A I & Murphy R I Pseudocysts of the pancreas Report of 31 cases Surg Gynes & Obst 88 219 229 1949
Iversen H G & Johansen Aa Cystadenocarcinoma pancreatis Acta Chir Scand to be published
Jensen F Solitær non parasitær Miltcyste Ugeskr Læg 116 1610–1612 1954
Korpássy B Die Basalzellemetaplasie der Ausführungsgange des Pancreas Virchows Arch f path Anat 303 359–373 1939
Mozan A A Cystadenoma of the pancreas Am J Surg 81 204–214 1951
Pool F H & Stillmann R G Surgery of the spleen p 109 D Appleton & Co New York 1923
Richmann A Acute pancreatitis Am J Med 21 246–274 1956
Schulte Heubach H J Über ein in die Milz metastasierendes Pseudomucinkystom der Ovarien Inaugural Dissert Bonn 1921
Snyder J W & Rezek P R Cysts of the spleen Southern Med J 36 263 268 1943
Zilstorff Pedersen K Cystic lienis non parasitica Ugeskr Læg 113 258–259 1951
Trolle E & Trolle D To sjældne splenomegaliformer Nord Med 18 761 766 1943

Acta path et microbiol scandinav 66 485–488 1966

Rheumatic Research Laboratory, University Institute of Pathological Anatomy (Head Professor G Teilum M D) and The Protein Laboratory (Head N Harboe, M D), University of Copenhagen Denmark

FRACTIONATION OF MOUSE SERUM PROTEINS

An Evaluation of two Methods with Special Reference to the γ-Globulins

By

P Ranløv

Received 18 viii 65

In immunological research involving the use of experimental animals the need for a qualitative and quantitative evaluation of the serum proteins often arises—especially the immunoglobulins These mostly expressed as γ-globulin In the major part of the literature this fractionation has been carried out by means of paper electrophoretic techniques (*Laurell, Laurell & Skoog* 1956) and this is true also for some recent reports in the field of experimental immunology (*Andersen & Bierring* 1964, *Andersen, Grunnet & Clausen* 1965

However, the paper electrophoretic techniques seem to suffer from certain drawbacks It is tedious and time-consuming (18 hours run), it demands at least 10 microlitres of serum, and the separation between the albumin and the α_1-globulin fractions is invariably poor Furthermore the albumin tailing added to the not insignificant background adsorption does compromise the accuracy of the quantitation Finally it must be borne in mind that the paper electrophoresis, originally designed as a clinical tool, was adapted to the fractionation of human sera in which the electrophoretic mobilities of the fractions not necessarily are identical with those of the corresponding murine bands

Kohn's electrophoretic techniques using cellulose acetate as supporting medium, seem to eliminate most of the disadvantages connected with paper electrophoresis (*Kohn* 1960) From these reasons we have found it justified to report our findings in a comparison of the two methods applied to serum from normal mice

MATERIAL AND METHODS

22 inbred C3H mice, 11 males Age 6–9 weeks (15–20 g) apparently healthy as judged by appearance and leucocyte and differential counts Blood was drawn in capillary tubes from the retroorbital plexus It was allowed to clot for one hour

These investigations have been supported by the *Danish League Against Rheumatism*

It ıs concluded that for purposes of separatıng murıne sera—especı ally when γ globulın determınatıons are consıdered ımportant—cellu lose acetate ıs far superıor to paper as supportıng medıum ın electro phoresıs

REFERENCES

Andersen S B & Bierring F Lymphoıd organs and normal gamma globulin in the rat Scıence *144* 1219 1220 1964

Andersen S B Grunnet I & Clausen J Splenectomy and serum proteıns in the guinea pig Acta path et microbiol scandınav *63* 317–318 1965

Harboe N Production of serum from small amounts of blood Scand J clin Lab Invest *8* 71 72 1956

Kohn J In Chromatographıc and Electrophoretıc Technıques Vol II Edıt Ivor Smıth William Heınemann London 1960

Laurell C B Laurell S & Skoog N Buffer composition ın paper electrophoresıs consıderations on ıts influence with specıal reference to the interactıon be tween small ıons and proteins Cl n Chem *2* 99 111 1956

Tıdstrom B Urinproteıner Thesis Copenhagen 1961

Acta path et microbiol scandinav 66 489 509 1966

The Virus Department of the Central Bacteriological Laboratory of Stockholm City, Stockholm Sweden

EXPERIMENTAL INFECTION WITH TICK-BORNE ENCEPHALITIS VIRUS IN CLETHRIONOMYS GLAREOLUS, APODEMUS FLAVICOLLIS, APODEMUS SYLVATICUS AND MUS MUSCULUS

1 *Virological Studies*

By

ZDENKA HEIGL[1] and GEROLF VON ZEIPEL

Received 16 ix 65

In a recent field survey (*Svedmyr et al* 1965) small mammals in regions of Sweden where tick borne encephalitis (TBE) is known to be endemic (*von Zeipel et al* 1959 a) were tested for the presence of neutralizing antibodies against TBE virus Species of small rodents dominating this material were selected for the following supplementary study of experimental infection with TBE virus in order to make possible a more correct evaluation of the rôle they play for the circulation of this virus in nature The species in question, Clethrionomys glareolus, Apodemus flavicollis, Apodemus sylvaticus and Mus musculus, have already been subjected to similar investigations although usually on a qualitative basis (*Solovjev* 1946 *Ernek et al* 1963 *Schindler & Krampitz* 1964, *Radda et al* 1964 a) It was, however, considered possible to obtain additional information using the same techniques for all species, and to compare the species in a quantitative way with regard to virus sensitivity, titre of viraemia and titre of antibody response

MATERIALS AND METHODS

Animals

White mice Commercially available white mice from the firm Anticimex, Stockholm Sweden were used througout This breed is claimed to be a continuation of the NMRI line (Naval Medical Research Institute U S A) The mice were 3 weeks old weighing about 8–10 g

---

This work was supported in part by the Swedish Medical Research Council (Project No S 356)

[1] WHO fellow 62R/Yug 23 On leave from the Department of Virology, School of Public Health "Andrija Stampar" Univ of Zagreb Yugoslavia

The skilful technical assistance of Miss *Siri Austrin* is gratefully acknowledged



Fig 1
Situation inland in the southern half of Sweden of Koping (1), Karlstad (2) and Mariestad (3) and of Stockholm (4) on the east coast

Wild rodents[1] Wild mice were trapped during the months of September October and November 1963 They mainly originated from the surroundings of the towns of Koping Karlstad and Mariestad situated inland in the southern half of Sweden (Fig 1)

The districts were selected so as to obtain as far as possible animals not exposed to TBE virus A special investigation had shown these districts to harbour cows with a relatively low incidence of antibodies against TBE virus (*von Zeipel et al* 1959 a) Later a small number of rodents were caught in the vicinity of Stockholm and a few animals were obtained from a breed at the vertebrate department of the Swedish Museum of Natural History Stockholm

In the field the animals were caught either in ordinary live traps for rats and mice or in Longworth traps The traps baited with cheese and a mixture of tallow and flour were emptied twice a day in order to get a maximal number of live animals

A total number of 177 animals were obtained For the tests the animals were arranged according to a preliminary classification by external characteristics (see *Van Den Brink* 1958) The final distribution into species given in Table 1 is based on the examination performed at autopsy after completion of the experiments

A discrepancy between the final classification and the preliminary one in the case of the Apodemus species is indicated by the figures in brackets The definite differentiation between A flavicollis and A sylvaticus was based on the length of the upper row of molar teeth (*Reinwaldt* 1957)

The distribution of the animals by age at the start of the experiments is given in Table 2

The age in the case of the two Apodemus species and in M musculus was determined by examination of the wearing of molar teeth (*Brealey* 1963 *Reinwaldt* 1963 and in the case of Cl glareolus according to the root length of the first lower molar (*Ma ak* 1963)

[1] The collaboration with the *Vertebrate Department of the Swedish Museum of Natural History* is highly appreciated We are grateful to Professor *Alf Johnels* head of the museum for valuable help and advice Our sincere thanks are due to Mr *Werner Berg* who was responsible for the collection of the animals and to Dr *Edwin Reinwaldt* who carried out the zoological classification of the animals and the determination of age and sex

TABLE 1
Distribution into Species

Species	Number of animals
Clethrionomys glareolus (*Schreber* 1780)	25
Apodemus flavicollis (*Melchior* 1834)	28 (preliminarily 25)
Apodemus sylvaticus (*Linné* 1758)	52 (preliminarily 55)
Mus musculus (*Linné* 1758)	72

TABLE 2
Distribution of Animals by Age

Species	No tested /total No	Age in months												
		2	3	4	5	6	7	8	9	10	11	12	17	18
Cl glar	25/25			1	2	2	12		5	1	2			
A flav	28/28						5	3	18				2	
A sylv	47/52				11		20	1	9				5	1
M musc	70/72	5	11	16	13	7	8	4	4			2		

TABLE 3
Distribution of Animals by Sex

Species	No tested /total No	Females	Males
Cl glar	25/25	11	14
A flav	28/28	12	16
A sylv	48/52	16	32
M musc	66/72	33	33

The distribution of the animals by sex is given in Table 3

All animals were kept in quarantine for several weeks before being used for the experiments They were caged individually and given a free supply of water and pelleted food together with fresh apples or carrots

The weight of each animal was recorded before the experiments began and then once a week

Temperature recordings were obtained rectally with an electrical thermometer (Type TF 3 Electrolaboratoriet Copenhagen Denmark) The bulb of the applicator was inserted about 20 mm (*Miya et al* 1963) Measurements were taken before inoculation with virus and repeated at each blood sampling

Blood was procured from the retro-ocular plexus of veins (*Halpern & Pacaud* 1951) with a Pasteur pipette rinsed in 0 1 per cent heparin in physiological saline Usually 0 5–1 ml of blood was obtained Additional heparin was added to constitute 1/10 of the volume of the sample After centrifugation in an angle centrifuge at 1500 rpm for 15 min the plasma was removed

The blood cells were washed once with 10–20 times their volume of phosphate buffered saline (PBS) and spun down again the supernatant was discarded

Plasma and red cells were stored in glass sealed ampoules in a dry ice box All blood samples obtained during the first three weeks after inoculation of virus were treated in this way From later samples only the plasma was saved this was stored at −30° C

Cold exposure experiments were carried out in a large refrigerator cabinet (1 3 m^3) ventilated with the aid of a water suction pump The temperature was recorded

at the
to
cm)
sawc
and
mach
and t
inoculation of
4 days
Cold expos
week
to +
cage
be
shi
cun
tha

virus

anaesthesia during all manipulations
autopsy was performed on all animals dying during the tests Brains were harvested under aseptic conditions and stored in a dry ice box until processed and tested as mentioned below The rest of the bodies were preserved in 10 per cent formalin The bodies of animals surviving at the end of the experiments were treated in the same way

Virus strains Two TBF virus strains were used one of Czech origin the other isolated in Sweden

The Czech Hypr strain was obtained in 1957 from Dr D Blaškovič Bratislava Czechoslovakia as a lyophilized mouse brain suspension
43rd intracerebral passage
more mouse brain
was used for the p
animals A 10 per c ... containing 10 per
I U of penicillin 100
by grinding with sand
3 minutes at 15000 rpm
centrifuge at 3000 rpm
extract showed no grov ... inoculated intracerebrally
with 0 02 ml of virus suspension

The Swedish
a dry
contai
Initial
for the present investigation
suspension was injected subc
of them succumbed Blood w
by the orbital meth
blood pooled
described for
highest virus
days after ino ... igation

Infectivity Serial dilutions of the various materials
from white mice and wild rodents
the

ice box

The techniques of neutralization and complement fixation are described in a following paper on serology (von Zeipel & Heigl) even though a few results are already given in this paper

Statistical Methods

Calculation of titres The LD_{50} end point of infectivity titrations was calculated according to *Karber* (1931) Except for Table 4 where the i c and s c values to be compared are recalculated to 1 ml all titre values refer to the dose actually in oculated (0 02 ml i c., 0 1 ml s c)

The significance of differences between frequencies was examined by the use of the χ^2 test Yates correction for continuity being applied throughout (see *Dixon & Massey* 1957)

The means of two populations of titres were compared by the Rank sum test (see *Dixon & Massey* 1957)

Calculations for the graphs Each point on the viraemia graphs represents the titre of one sample from an infected animal All animals inoculated with full doses of virus developed serological evidence of infection This was not the case with animals inoculated with 33 s c LD_{50} of virus or less however Provided that at least one sample from an animal was virus positive and or that there was sero logical evidence of virus infection virus negative specimens from the same animal have been ascribed a maximal titre value after extrapolation from the dilution series used in the test Extrapolation was performed in the following way If the dilution series 1/6 1/15 1/150 1/1500 was negative dilution 1/2 4 (2 5 fold step) was assumed to be positive (all tubes) The titre according to *Karber* would then be —0 6

The trend lines of virus titres are drawn through points representing geometric mean values In calculating the geometric mean negative results were included as maximal titre values to the extent mentioned above

SCHEME OF EXPERIMENTS

General plan The experiments with the Hypr and Hallboda strains were carried out during separate periods the former strain being tested first Because of the limited number of animals available it was not possible to make a comparison of both strains in all four animal species The tests were run at room temperature and at +4° C (2 5° C) A fixed dose of 100 subcutaneous LD_{50} white mouse units of virus was aimed at It was inoculated subcutaneously in 0 1 ml In the Hallboda experiment an attempt was also made to assess the susceptibility at room temper ature of two species to minimal doses of virus

In both experiments the virus strain was simultaneously titrated in white mice by the subcutaneous as well as the intracerebral route

The disposition of the wild rodents available for these experiments may be seen in Tables 7 and 12

The animals of each experimental group were divided into three subgroups efforts being made to get an even distribution by weight Preinoculation blood was drawn from all animals After inoculation the groups were bled alternately every other day for three weeks in the Hypr experiment and for two weeks in the Hallboda ex periment thus the shortest interval between the sampling of blood from one animal was 6 days The majority of animals were then bled at longer intervals for a total of 42 days some in the Hallboda experiment for as much as 175 days

In order to check that the animals did not develop anaemia as a result of the repeated samplings the content of haemoglobin was estimated in the first and last samples of 4–5 animals in each group In none of the animals tested were signs of anaemia detected and the variations in haemoglobin content in the same animal were found to be within the limits of experimental error

The animals were inspected twice a day for signs of disease

RESULTS

Pathogenicity of Strains Hypr and Hallboda in White Mice

The difference between LD_{50} titres after central and peripheral in oculation into white mice was of about the same order for both strains Hypr and Hallboda although the latter had never been passed intra cerebrally in the laboratory (Table 4) This difference has been re

ported to be a marker characteristic for a given strain (*Shah et al* 1962 *Mayer* 1964)

TABLE 4

Intracerebral and Subcutaneous LD_{50} Titres per ml of Strains Hypr and Hallboda in White Mice

	i c	s c	Difference i c/s c
Hypr 46th brain passage	7 8 mean 7 5 7 2	5 9 mean 5 8 5 7	1.9 mean 1 7 1 5
Hallboda 3rd blood passage	6 8 mean 6 4 5 9	4 9 mean 4 9 4 8	1 9 mean 1 5 1 1

The virus dose as determined in white mice used for infection of wild rodents turned out to be 75 subcutaneous LD_{50} units of strain Hypr and 330 similar units of strain Hallboda corresponding to 3700 and 10000 intracerebral units respectively

Infection of Wild Rodents

Clinical Data

General condition There was no obvious impairment of the general behaviour appetite or change in weight of any animal after infection except for some of those listed below as paralytic or dead

Temperature measurements In the experiment with strain Hypr the rectal probe was only inserted 10 mm resulting in very irregular and non reproducible measurements from which no conclusions could be drawn

The analysis of measurements obtained from the Hallboda experiment when the applicator was properly inserted (20 mm) does not indicate a rise in temperature after inoculation of virus in any of the four animal species tested This is illustrated in Table 5, in which the arithmetical means of measurements during the first half of the observation period are given for animals at room temperature

TABLE 5

Mean temperature Recordings on Indicated Days

	3	5	7	9	12	19
Cl glareolus	37 8	37 9	37 2	37 0	37 3	37 8
A flavicollis	39 3	39 4	39 3	39 9	39 4	39
A sylvaticus	38 3	38 7	38 5	38 6	38 4	38 [illegible]
M musculus	38 5	38 0	38 5	38 3	38 0	38

Paralysis Three animals kept at room temperature developed paralysis Two of the animals had been inoculated with Hypr and one with Hallboda virus Short clinical notes on these animals are given below, virological and serological data are presented in Table 6

TABLE 6

Virological and Serological Data of Paralytic Animals

Species	Day of bleeding	Virus in brain	Virus titre of plasma	CF titre of plasma	NT titre of plasma
M musc No 20	1		—	—	
	7		1 1	—	
	13		—	12	—
	21			12	24
	35			24	96
	42	*		24	576
M musc No 25	3		—		
	9		—	6	—
	15		—	48	96
	25			48	384
	42	*		96	768
Cl glareolus No 4	5		3 5	—	
	12		—	12	
	23			96	2662
	24	—			

* not tested healthy animal — = negative result

TABLE 7

Animals Succumbing after Infection with Hyp and Hallboda Viruses

Virus	Animal species	Temperature conditions °C	No of animals inoculated	No of control animals
Hypr	*A sylvaticus*	22	5/15	0/3
	"	4	3 8	0/2
	M musculus	22	3/18	0 4
	"	4	10 12	2/3
Hallboda	*Cl glareolus*	22	2/12	
	"	4	3/13	
	A flav collis	22	0/15	
	"	4	0/13	
Titration of Hallboda virus	*A sylvaticus*	22	0/24	
	M musculus	2?	1/35	

Numerator Number of dead animals Denominator Total number of animals

Hypr Experiment

M musculus No 20 The animal showed weakness of left front and hind legs 10 days after inoculation of virus The gait was uncertain and the animal often rolled over on the left side No complete paralysis developed The general condition of the

TABLE 8

Data for Animals Succumbing after Inoculation of Hypr Virus

Species Temperature	Animal No	Day of death	Virus titre of brain	Day of blood sampling	Virus titre of plasma	NI titre of plasma	Probable cause of death autopsy findings
A sylv +4° C	5	1					Blood sampling
	6	5		5	1 1		Blood sampling
	1	7	—	3	1 5		Unknown
A sylv +22° C	20	3	—	3	1 0		Blood sampling
	13	3	—	3	1 1		Blood sampling
	23	7	—	1 7	2 3 —		Blood sampling
	12	17	1 7	5 11 17	—	 24 24	Blood sampling
	19	17	3 1	5 11	—	 —	Blood sampling
	7	2					Blood sampling
	4	2	—	1	0 7		Unknown

	11	8	0 7	3	—		Unknown
	5	8	2 3	3	1 3		Unknown
	12	10	3 9	5	1 5		Unknown, Subcapsular necrosis of left kidney
	16	10	2 1	5	1 0		Unknown
M musc +4° C	8	11	4 5	5 11	2 7	 24	Blood sampling
	10	12	2 3	1 7	— 1 1		Unknown
	9	15	4 5	5 11	3 1 —	 24	Unknown
	2	20	0 9	3 9 15	2 7 — —	 6	Small intestine filled with blood Thermometer injury?
	24	7	0 7	1 7	— —		Blood sampling
M musc +22° C	29	15	3 7	5 11	2 3 —	 —	Pneumonia and necrosis of left lung
	23	17	0 7	5 11 16	1 5 —	 48 48	Blood sampling

TABLE 9

Data for Animals Succumbing after Inoculation of Hallboda Virus

Species Temperature	Animal No	Day of death	Virus titre of brain	Day of blood sampling	Virus titre of plasma	CF titre of plasma	Probable cause of death, autopsy findings
Cl gl +4° C	18	7	1 5	1	—	—	Blood sampling
				7	—	12	
	6	27	—	1	0 7	—	Unknown
				7	—	12	
				14		48	
	24	27	—	1	1 1	—	Unknown
				7	—	12	
				14		24	
Cl gl +22° C	25	20	—	1	0 7	—	Blood in the Bladder
				7	0 8	12	Thermometer injury?
				14		96	
	7	22	1 1	1	1 0	—	Unknown
				7	0 7	—	
				14		12	
M musc +22° C	45	24	1 9	1	1 5	—	Small intestine
				9	—	96	filled with blood
				19		96	Thermometer injury?

animal was not markedly impaired After 10 days of illness the animal recovered successively and at the end of the experiment was registered as healthy

M musculus No 25 The animal developed complete paralysis of the right hind leg 12 days after inoculation of virus On the following day both hind legs were paralytic This condition lasted for three days after which the left leg could be used again A slight improvement in the right leg was also noticed, but this leg remained paralytic until the experiment was finished The general condition of the animal was good and it climbed to the cover of the cage with the front legs even when both hind legs were paralysed

Hallboda Experiment

Cl glareolus No 4 The animal developed complete paralysis of both hind legs 22 days after inoculation of virus The general condition of the animal was obviously impaired The animal was killed two days after onset of the illness since it could hardly get to its feet and had difficulty in reaching food and water The virological tests for the presence of virus in brain and spinal cord were negative

Deaths The number of animals which succumbed in the Hypr and Hallboda experiments is recorded in Table 7

Details about the dead animals are given in Tables 8 and 9

Only two out of the 27 animals recorded in Tables 8 and 9 showed symptoms of illness (ruffled fur and weakness) during some few days before death In both animals severe pathological changes were found at section, one, (Table 8, *M musculus* No 29, Hypr), having pneumonia with central necrosis completely involving the left lung, and the small intestines of the other (Table 9, *M musculus* No 45, Hallboda) being filled with blood probably originating from injury to the gut wall caused by the thermometer bulb The remaining animals were healthy and in good condition at the last inspection, which, however, in may cases was undertaken up to 18 hours before death Blood sampling and/or anaesthesia was recorded as a possible cause of death only for animals dying in immediate conjunction with this procedure (10 animals) or on the same day (2 animals)

From Tables 8 and 9 it is evident that with a single exception all deaths connected with blood sampling occurred at an early stage of the Hypr experiment This test, as mentioned above, was carried out before the Hallboda experiment

Table 10 illustrates that in 10 out of 11 animals kept at room temperature a probable cause of death could be found In 10 out of the 16 animals kept at +4° C, on the other hand, no obvious cause of death could be found Not less than 7 of these 10 cases belonged to the *M musculus* group of the Hypr experiment Two uninoculated control animals of this species also died at +4° C The species was not exposed to cold in the Hallboda experiment

The results of virus isolation tests on the brains of dead animals are summarized in Table 11 regardless of whether the animals had been kept at room temperature or in the cold No special precautions were taken to wash the brain material free of blood before the preparation of suspensions in any of these cases Although a low titre of viraemia

TABLE 10
Summary of Causes of Death

Virus Species Temperature	No of deaths	Probable cause of death			
		Blood sampling anesthesia	Bleeding from thermometer injury	Pneumonia	Unknown cause of death
Hypr					
A sylv +4° C	3	2			1
A sylv +22° C	5	5			0
M musc +4° C	10	2	1		7
M Musc +22° C	3	2		1	0
Hallboda					
Cl glar +4° C	3	1			2
Cl glar +22° C	2		1		1
M musc +22° C	1		1		0
Total	27	12	3	1	11

TABLE 11
Virus Titres of Brains from Animals that Succumbed after Inoculation of Hypr and Hallboda Viruses

Titer of brain	2	3	7	8	10	11	12	15	17	20	22	24	27
4 5						c		c					
3 9					c								
3 7								c					
3 1									b				
2 9							c						
2 3				c									
2 1					c								
1 9												*c*	
1 7									b				
1 5			*a*										
1 1											*a*		
0 9										c			
0 7			c	c					c				
neg	c	bb	bb							*a*			*aa*

…) after inoculation when the brain was removed

…ters a *C glareolus* b *A sylvaticus* c *M musculus*
…ters in italic refer to the Hallboda experiment

Fig 2

Viraemia in *Apodemus sylvaticus* Hypr experiment
Virus dose 75 subcutaneous LD_{50} for white mice
● positive animal at +22° C ○ negative animal at +22° C.
▲ positive animal at + 4° C △ negative animal at + 4° C

was found in the animals which died on day 2 and 3, virus was not obtained from the brain until on the 7th day In specimens originating from day 8 to day 17, virus was regularly recovered and moreover, the highest titres reached Virus was present in the brain of *A sylvaticus*, *Cl glareolus* and *M musculus* as late as on days 17 22 and 24 respectively

The only virus isolation attempts on brain material from animals surviving the experiments were performed on *Cl glareolus* and *A flavicollis* inoculated with *Hallboda virus and kept at +4° C for 42* days after inoculation As they were all negative in spite of precautions to wash the brain fragments free of antibody before disintegration, no more isolation attempts were made with the brains of other surviving animals

Virological Data

Hypr experiment Viraemia following s.c inoculation of 75 s.c LD_{50} of Hypr virus in *A sylvaticus* and in *M musculus* is recorded in Fig 2 and Fig 3, respectively

Within each species there is no obvious difference, irrespective of whether the animals were kept at room temperature or at +4° C Consequently groups of animals tested at different temperatures were pooled for comparison of appearance, duration and peak titre of viraemia in the two species

In general the curve of viraemia of *A sylvaticus* is displaced in

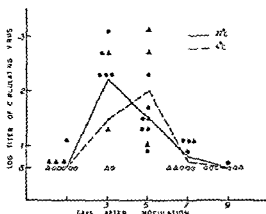


Fig 3
Viraemia in *Mus musculus* Hypr experiment
Virus dose 75 subcutaneous LD_{50} for white mice
● positive animal at +22° C ○ negative animal at +22° C
▲ positive animal at + 4° C △ negative animal at + 4° C

time for about two days in comparison with the curve of *M musculus* Thus almost all *A. sylvaticus* but less than half of *M musculus* were positive on the first day after inoculation of virus, the titres recorded in the latter species being in addition significantly lower The descending part of the curve will furthermore reach the approximate 50 per cent point for infectious specimens on the 5th day in *A sylvaticus* and on the 7th day in *M. musculus* Of the latter species one animal was positive even after 9 days The peak of the curve recorded in *M musculus* on the 3rd to 5th day was slightly higher than the peak encountered in *A sylvaticus* at the beginning of the curve

Hallboda experiment Viraemia following inoculation of 330 s c LD_{50} of Hallboda virus in the species *Cl. glareolus*, *A flavicollis* and *Mus musculus* is recorded in Figs 4, 5 and 6, respectively The first two species were tested at room temperature and at + 4° C *Mus musculus* at room temperature only

Also in these experiments similar results were obtained at different temperature conditions Equivalent groups of animals are consequently pooled as mentioned above

Between the species *Cl glareolus*, *A flavicollis* and *M musculus* no significant differences were found in appearance and duration of viraemia A high percentage of the animals were positive on the first day of sampling although titres were minimal With one exception virus was found in all specimens originating from day 3 and day 5 On the 7th day about half of the specimens were positive in *Cl glareolus* and in *A flavicollis* At the same time virus was recovered from the majority of *M musculus* Again one animal of the latter species was positive on day 9 Peak titres were reached in *C glareolus* and in *A flavicollis* on the 3rd day and in *M musculus* on the 5th day, the mean value for

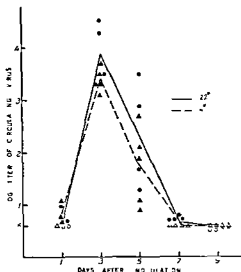


Fig 4

Viraemia in *Clethrionomys glareolus* Hallboda experiment
Virus dose 330 subcutaneous LD_{50} for white mice
● positive animal at +22° C. ○ negative animal at +22° C
▲ positive animal at + 4° C. △ negative animal at + 4° C

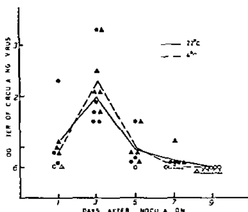


Fig 5

Viraemia in *Apodemus flavicollis* Hallboda experiment
Virus dose 330 subcutaneous LD_{50} for white mice
● positive animal at +22° C. ○ negative animal at +22° C
▲ positive animal at + 4° C. △ negative animal at + 4° C.

A flavicollis being significantly lower than for the other species (level of significance 1 per cent and 0 1 per cent for *M musculus* and *Cl glareolus*, respectively, according to the Rank sum test) The peak titres recorded for *M musculus* on days 3 and 5 are significantly higher

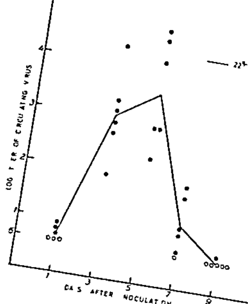


Fig 6

Viraemia in *Mus musculus* Hallboda experiment
Virus dose 330 subcutaneous LD_{50} for white mice
● positive animal at +22° C. ○ negative animal at +22° C

in the Hallboda test than in the Hypr test (level of significance 0 1 cent according to the Rank-sum test)

Estimation of the LD_{50} point in the titration tests A summary of titration of strain Hallboda in *A sylvaticus* and *M musculus* is given in Table 12 The results are based on virological and/or serologic evidence of virus infection Apparently one infectious (ID_{50}) unit of virus in *A sylvaticus* corresponds to one lethal (LD_{50}) unit of virus in white mice, and about 5 (incomplete titration) lethal units are required for infection of *M musculus*

TABLE 12

Titration of strain Hallboda in

Virus dose	*A sylvaticus*	*M musculus*
330 s c LD_{50}		
33	4/4	18/18
3 3	6/6	7/8
0.3	5/6	4/9
0 03 "	1/4	
	0/4	

Numerator Number of animals showing viraemia
Denominator Total number of animals inoculated

One "*A sylvaticus*" not recorded in Table 14 proved at the final classification to belong to the *A flavicollis* species The animal deve

loped \iraemia after inoculation with 0 3 LD_{50} of \irus Thus *A flavicollis* too may be infected by minimal doses of \irus

Demonstration of virus in the blood cell fraction All information on \iraemia given in Figs 2–6 is based on titration of plasma The washed blood cells corresponding to the plasma specimens from the 9th, 12th and 14th day of the Hallboda experiment were tested for the presence of \irus after one cycle of freezing and thawing and dilution 1/4 The results are summarized in Table 13

TABLE 13

Presence of \irus in Plasma and in Blood Cell Fraction

Species	s c LD_{50} \irus dose	Plasma day 9	Blood cells day 9	Blood cells day 12	Blood cells day 14
Cl glareolus	330	0/8	0/8	*1/8*	0 8
A flavicollis	330	0/10	0/9	0/8	0/8
A sylvaticus	*330*	*0/1*	*0/1*	*0/2*	*0/1*
"	33	0/2	*2/2*	0/2	0 2
"	3	0/2	0 2	0 2	0/1
M musculus	330	1/6	*5 6*	*1/6*	0 6
"	33	1/3	*3 3*	0/2	0/2
"	3	0 3		*1/1*	0/3

\umerator \umber of positive specimens
Denominator Total number of specimens

In all species except *A flavicollis* \irus could be demonstrated for a longer time in the blood cell fraction than in plasma The difference in *M musculus* between positive isolations from cell extracts and plasma of the 9th day is probably significant ($P<0\ 02$, χ^2 test)

It should be mentioned that most animals with late positive virus finding in the cell fraction had by this time demonstrable neutralizing antibody in the plasma (10 out of 10 *M musculus*, 1 out of 2 *A sylvaticus*) Actually, \irus was also isolated from two of the plasma specimens with neutralizing antibody from *M musculus*, it seems possible that these specimens contained small amounts of infectious cells or cell fragments, however

DISCUSSION

In the majority of animals tested the infection with TBE \irus seems to have run an inapparent course, no fever or any other clinical symptoms of disease being noticed The only species, however, in which all individuals remained free from symptoms was *A flavicollis* In *Cl glareolus* and *M musculus*, cases of paralysis occurred In the latter species as well as in *A sylvaticus*, some animals died The role played by the TBE infection in the cases of paralysis and death is unclear and will need some further comment

The aetiology of paralysis may not necessarily be the same in all

three cases TBE virus infection is only one of several possibilities. Activation of a latent virus on the analogy of Theiler virus must also be considered, as well as mechanical trauma of the spinal cord in handling the animals for instance during blood sampling. All three possibilities remain open, there was actually no evidence of virological cause. It must, however, be admitted that even a positive TBE virus isolation had not proved this virus to be of aetiological importance, for it was shown to be present in the CNS for a relatively long time in other animals investigated.

Most cases of death among the animals occurred during the viraemic stage or during a period when the animals were shown to harbour virus in their brains. The TBE infection may thus have contributed to the lethal outcome. However, in more than half of the cases there was a probable cause of death, i.e. animals with severe pathological changes at autopsy and those which died during blood sampling. In the latter group an imperfect technique in sampling and/or anaesthesia seems to have been mainly responsible for the deaths, for these were met with almost exclusively during an early stage of the first experiment. A decreased resistance resulting from TBE infection and blood sampling may also have contributed to the high lethality of unknown cause in animals exposed to cold. However, at least in the case of *M. musculus* where 7 out of 10 infected animals died, this may be explained by the influence of cold alone since also 2 of 3 control animals succumbed. As mentioned above, none of the animals which died on exposure to the cold showed any previous signs or symptoms of disease.

Thus in the present investigation no evidence was found for an increased pathogenicity of TBE virus in wild rodents after cold exposure. Correspondingly no influence on either appearance, duration or titre of viraemia was demonstrable. Such treatment is, however, known to influence the host-parasite interaction in some other systems. An increased death rate on cold exposure has, for instance been reported in white mice infected with Coxsackie B 1 virus (*Boring et al.* 1956, *Walker & Boring* 1958) and in rabbits infected with myxoma virus (*Marshall* 1959).

No published information on the experimental infection of *A. sylvaticus* with TBE virus has been available with the exception of a brief statement by *Radda et al.* (1964 b). Reports on *A. flavicollis* are in agreement with our results, no symptoms of disease being observed by *Ernek et al.* (1963) or by *Schindler & Krampitz* (1964). These two groups of workers also found adult *Cl. glareolus* to be highly resistant to clinical disease, whereas very young animals developed a fatal TBE infection (*Schindler & Krampitz* 1964). In *M. musculus* more varying results have been reported. The species, according to *Schindler & Krampitz* (1964), is not sensitive to TBE virus provided the virus dose inoculated does not exceed 1 million i.c. LD_{50}. A still higher dose was probably used by *Solovjev* (1946), resulting in the death of the animals

between the 5th and 10th day Since he used a Far Eastern strain of RSSE virus his results may not be comparable however *Radda et al* (1964 a) working with a tissue culture line of strain Hypr and two strains of *M musculus* reported that only some individuals of one of the strains survived following inoculation of a virus dose comparable to the one used in the present investigation without showing signs of illness and that the virus caused disease and/or death in all individuals of the other strain the origin of which was however obscure These authors as well as Schindler & Krampitz therefore discuss the possibility of a varying sensitivity of different strains of *M musculus*

The presence of virus in rodent brains harvested several weeks after inoculation of virus is known from other investigations Thus *Solovjev* (1946) was able to demonstrate RSSE virus in the brain of *A flavicollis* 20 days after infection Moreover *Ernek et al* (1963) reported TBE virus to be present in the brain of *Cl glareolus* on the 28th day *Radda et al* (1964 a) however were unsuccessful in isolating virus from brains of *M musculus* 9 to 28 days after inoculation with the exception of a few mice showing signs of illness before being killed None of the authors mentioned give any quantitative data however In the present investigation only brains from animals which died were tested It is not known whether surviving animals including all representatives of *A flavicollis* were also virus positive during the same period However in the light of Solovjevs and Erneks findings this does not seem improbable

The duration of the viraemic period in the different species as demonstrated in the present investigation seems to be in good agreement with published reports *Ernek et al* (1963) using heparinized blood were able to demonstrate viraemia in *Cl glareolus* and *A flavicollis* as late as the 9th day *Schindler & Krampitz* (1964) found virus to be present in the blood for 8 days in *Cl glareolus* and *M musculus* *Radda et al* (1964 a) on the other hand report viraemia of only 4 to 5 days duration in *M musculus* with the exception of animals that died later from encephalitis

The possibility of demonstrating virus with greater ease and for a somewhat longer time using the cell fraction instead of whole blood or plasma is known for some other viruses (for references see *Gresser & Chany* 1963) The method also proved to be of value in the present investigation Whether or not a repeated washing of the cells might further increase the sensitivity is not known

The highest titre given by *Ernek et al* (1963) for viraemic specimens of *Cl glareolus* and *A flavicollis* was $10^{1.5}$ mouse LD_{50} per 0 03 ml In all four species tested in the present investigation animals were found having titres of about 2 to more than 3 logs higher

The higher virus titre obtained in *M musculus* with strain Hallboda may reflect a difference between this strain and the Hypr virus Whether or not the difference in titre may be attributed to the strain *per se* or

to the difference in passage story or size of dose cannot be decided upon

The demonstration that the species *A flavicollis*, *A sylvaticus* and *M musculus* are as susceptible to TBE infection as white mice and the fact that individuals of all the species tested may reach high virus titres in the blood indicates the importance of these animal species for the circulation of TBE virus in nature Considering the duration and titre of viraemia, the population of *M musculus* here tested would have the prerequisites for being the most potent disseminator of TBE virus of the species investigated (*Cl glareolus* being omitted from the comparison because of unknown susceptibility to minimal doses of virus)

SUMMARY

The pathogenicity, following subcutaneous inoculation of two TBE virus strains, *one never being brain passed, was tested in a study involving* 177 wild rodents of the following species *Cl glareolus*, *A flavicollis*, *A sylvaticus* and *M musculus* Most animals were inoculated with a dose of about 100 s c LD_{50} for white mice

In the majority of the animals of all species the infection was inapparent The aetiology of 3 cases of paralysis is discussed, as well as the cause of death in 27 animals, 10 of which succumbed during cold exposure, and 12 in direct connection with anaesthesia and blood sampling with no other obvious cause

The presence of TBE virus was almost always demonstrated in the brain of animals that died from 1 to 3 weeks after inoculation of virus

The duration of viraemia based on titration of plasma was found to last for 5 to 9 days, the shortest period being recorded in *A sylvaticus* and the longest in *M musculus* Virus could however, be recovered from cell extracts for a still longer time High virus titres of the blood were recorded in animals of all species, the maximal values per 0 02 ml being $10^{3.3}$ in *A flavicollis*, $10^{4.1}$ in *A sylvaticus*, $10^{4.5}$ in *Cl glareolus* and $10^{4.7}$ in *M musculus* The mean titres of viraemia were, however higher *in M musculus and Cl glareolus than in the Apodemus species*

The species *A flavicollis*, *A sylvaticus* and *M musculus* were infected by about one subcutaneous LD_{50} unit of virus for white mice

REFERENCES

Boring W D ZuRhein G M & Walker D L Factors influencing host virus interactions II Alterations of Coxsackie virus infection in adult mice by cold Proc Soc Exper Biol Med 93 273 277 1956

Breakey D R The breeding season and age structure of feral house mouse populations near San Francisco Bay California J Mammalogy 44 153 168 1963

Van Den Brink F H Zoogdierengids Elsevier 1958 Swedish translation Alla Europas Daggdjur Albert Bonnier Stockholm 1958

Dixon W J & Massey F J Introduction to elementary statistical analysis MacGraw Hill Inc New York 1957

Ernek E Kozuch O Lichard M Nosek J & Albrecht P Experimental infection of *Clethrionomys glareolus* and *Apodemus flavicollis* with tick borne encephalitis virus Acta Virol 7 434-436 1963

Gresser I & Chang Ch Isolation of measles virus from the washed leucocytic fraction of blood Proc Soc Exper Biol Med *113* 695–698 1963

Halpern B N & Pacaud A Technique de prélèvement d echantillons de sang chez le petits animaux de laboratoire par ponction du plexus ophthalmique C R Soc Biol *145* 1465–1466 1951

Kärber G Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche Arch f exper Path u Pharmakol *162* 480–483 1931

Marshall I D The influence of ambient temperature on the course of Myxomatosis in rabbits J Hyg 57 484–497 1959

Mayer V Study of the virulence of tick borne encephalitis virus II Pathogenetic properties of two variants of a single virus strain Acta Virol *8* 14–21 1964

Ma ák V Notes on the dentition in *Clethrionomys glareolus* Schreber 1780 in the course of postnatal life Saugetierkundliche Mitteilungen *11* 1 11 1963

Radda A Pret mann G & Kovac W Experimentelle Infektion von *Mus musculus* mit Fruhsommer Meningoenzephalitis (Tick borne Encephalitis) Virus Zbl f Bakt I Orig *193* 285 292 1964 a

Radda A & Pretzmann G Einfluss der Hibernierung auf die Virusvermehrung nach experimenteller Infektion des Ziesels (*Citellus citellus*) mit Fruhsommer Meningoenzephalitis (Tick borne Encephalitis) Virus Zbl f Bakt I Orig *193* 413 420 1964 b

Reinwaldt F Studien am Schadel der swedischen Waldmause *Apodemus sylvaticus* (Linné 1758) und *Ap flavicollis* (Melchior 1834) Saugetierkundliche Mitteilungen 5 100–103 1957

Re nwaldt E Uber zeitlebens wachsende Molaren der *Murinae* (Mammalia Muridae) Ark Zool *17* 561 571 1965

posium of the Czechoslovak Academy of Sciences Biology of viruses of the tick borne encephalitis complex II 1960 Academic Press Inc New York 3 p *303–310 196?*

Solovjev V D in *Silber A L & Solovjev V D* Far Eastern tick borne spring summer (spring) encephalitis American Review of Soviet Medicine Special Supplement American Soviet Medical Society New York. U S A., 1946

Svedmyr A Johnels A Brink P von Zeipel G Heigl Z Lundholm B & Zetterberg B Fastingburet encefalitvirus i Sverige Ekologiska undersokningar Nordisk Medicin 73 188 190 1965

Walker D L & Boring W D Factors influencing host virus interactions III Further studies on the alteration of Coxsackie virus infection in adult mice by environmental temperature J Immunol *80* 39 44 1958

von Zeipel G Svedmyr A & Zetterberg B The geographical distribution in Sweden of viruses belonging to the Russian spring summer louping ill group Arch ges Virusforsch *9* 449–459 1959 a

von Zeipel G Isolation of viruses of the Russian spring summer encephalitis louping ill group from Swedish ticks and from a human case of meningo encephalitis Arch ges Virusforsch *9* 460–469 1959 b

von Zeipel G & Heigl Z Experimental infection with tick borne encephalitis virus of *Clethrionomys glareolus Apodemus sylvaticus Apodemus flavicollis* and *Mus musculus* II Serological studies Acta path et microbiol scandinav In press

Acta path et microbiol scandinav 66, 510-518, 1966

The National Institute of Public Health Virus Department B, Oslo, Norway
(Head A Harboe M D)

A URONIC AND SIALIC ACID FREE CHICK ALLANTOIC MUCOPOLYSACCHARIDE SULPHATE WHICH COMBINES WITH INFLUENZA VIRUS HI-ANTIBODY TO HOST MATERIAL

2 *Chemical Composition*

By

GUNNAR HAUKENES, ARILD HARBOE and
KAREN MORTENSSON EGNUND

Received 1 ix 65

The purification of the chick allantoic antigen, which combines with antibody to influenza virus host material (12 13), was described in a foregoing publication (14) The resulting purified substance reacted to high titres serologically and was antigenic on intravenous injection in rabbit It was found to be composed mainly of neutral sugar, hexosamine and ester sulphate The protein or peptide moiety was small and the substance was free of sialic acid and uronic acid

The present article deals with the finer chemical composition of the purified host antigen Some experiments on modifications of the purification procedures have also been included

MATERIAL AND METHODS

The HIB (Haemagglutination Inhibition Blocking) test was carried out as described in earlier publications (13, 14)

Host antigen Two purified preparations of the host antigen were examined in the chemical analyses reported in this article One preparation was the same as described in the previous article (14) The other substance was purified by ion exchange chromatography only since acid precipitation of the host antigen from the concentrated allantoic fluid gave an unsatisfactory yield (cf below under Experimental) The two preparations showed rather similar chemical composition

Ion exchange chromatography DEAE Sephadex columns for preparation of the host antigen were prepared and run as described before (14) DEAE cellulose (Serva grade p a) columns were prepared according to the manufacturer s directions and were run by stepwise or gradient elution along the same lines as the DEAE Sephadex columns The host antigen was eluted from the DEAE cellulose columns at a lower NaCl molarity than from DEAE Sephadex Zerolit 225×8 and Dowex

---

The authors are indebted to Mr A Bye Hansen Miss T Folland and Mrs L Wang for skilled technical assistance

50 × 8 columns > 200 mesh were prepared in the H form and run as described by *Crumpton* (6) for separation of amino sugars according to *Gardell* (11).

Neutral sugars (27), *methyl pentoses* (7), *total hexosamines* (2) and *proteins* (16) were assayed essentially as described in the former article (14). Galactose served as a standard in the orcinol reaction because this hexose was the only one found to be present. The reported hexosamine contents have for the same reason been calculated as the N-acetyl derivatives.

Ester sulphate was determined as before by the turbidimetric method of *Dodgson & Price* (8) and also by a colorimetric method (9), both after hydrolysis with 2N HCl at 105° C for 5 hours. Nonhydrolyzed samples of the host antigen were included as controls.

N-acetylhexosamines were determined by the method of *Reissig et al.* (22).

Electrophoresis. Free boundary electrophoresis was carried out at the Norwegian Institute of Seaweed Research, Trondheim, through the courtesy of cand. ing. *Bjørn Larsen.* Ascending-descending boundary electrophoresis was performed with an 1 per cent solution of the purified host antigen in 0.01 N HCl containing 0.05 M NaCl applying a current of 10 mA for 60 minutes.

Paper electrophoresis was performed with Oxoid cellulose acetate strips 20 × 5 cm in a Shandon Universal electrophoresis apparatus with a power supply (LKB Type 3371 A) equipped for stabilization of voltage. The electrophoresis was run at 4° C and with about 20 V per cm.

Infra red spectroscopy was carried out at the Central Institute for Industrial Research, Oslo, with the purified antigen preparation incorporated into a KBr disc.

Specific optical rotation was measured with a *Hilger & Watts* polarimeter Type M 412.

Paper chromatography was carried out by the descending method on Whatman No. 1 filter paper. Acid hydrolysis. For identification of sugars the substance was hydrolyzed with 4N HCl at 100° C for 4 hours, and with 6N HCl at 110° C for 16 hours for amino acids. The special hydrolysis conditions applied for demonstration of fucose, N-acetylhexosamines and sulphated monosaccharides will be reported below under Experimental. The hydrochloric acid was removed either by repeated evaporations of the hydrolysate or by neutralization, evaporation to dryness and extraction with pyridine. Removal of the hydrochloric acid by evaporation to dryness caused considerable destruction of the fucose and also some destruction of the galactose.

Solvent systems:

A. Pyridine-ethyl acetate-water (1 : 3.6 : 1.1), organic phase (19).
B. n-Butanol-ethanol-water (3 : 1 : 1) with 1 per cent cethyl pyridine chloride (CPC) (21).
C. n-Butanol-acetic acid-water (50 : 12 : 25), organic phase (23).
D. n-Butanol-acetic acid-water (4 : 1 : 5), organic phase (6).
E. Phenol-water (4 : 1, w/v) (5).
F. Phenol-water (4 : 1, w/v) with 1 per cent ammonia added at the bottom of the jar (5).

Developing reagents:

1. Ninhydrin 0.4 per cent in n-butanol with 10 per cent phenol and acidified with [illegible]
[illegible] for individual amino acids
[illegible] oxyamino acids
[illegible] for reducing sugars
[illegible] sugar alcohols (4).
6. The *Elson-Morgan* method for amino sugars as modified for paper chromatography by *Partridge* (20).
7. The same as reagent 6 except that acetyl acetone was omitted and the condensation was carried out with alkali only, as specific for N-acetylhexosamines (20).

The N-acetylglucosamine-6-sulphate reference substance was kindly supplied by Dr. *F. Meegan*, Duke University Medical Center, Durham, N. C., U. S. A.

Acta path et microbiol scandinav 66 510–518 1966

The National Institute of Public Health Virus Department B, Oslo, Norway
(Head A Harboe, M D)

A URONIC AND SIALIC ACID FREE CHICK ALLANTOIC MUCOPOLYSACCHARIDE SULPHATE WHICH COMBINES WITH INFLUENZA VIRUS HI-ANTIBODY TO HOST MATERIAL

2 *Chemical Composition*

By

GUNNAR HAUKENES, ARILD HARBOE and
KAREN MORTENSSON-EGNUND

Received 1 ix 65

The purification of the chick allantoic antigen, which combines with antibody to influenza virus host material (12, 13), was described in a foregoing publication (14) The resulting purified substance reacted to high titres serologically and was antigenic on intravenous injection in rabbit It was found to be composed mainly of neutral sugar, hexosamine and ester sulphate The protein or peptide moiety was small, and the substance was free of sialic acid and uronic acid

The present article deals with the finer chemical composition of the purified host antigen Some experiments on modifications of the purification procedures have also been included

MATERIAL AND METHODS

The HIB (Haemagglutination Inhibition Blocking) test was carried out as described in earlier publications (13 14)

Host antigen Two purified preparations of the host antigen were examined in the chemical analyses reported in this article One preparation was the same as described in the previous article (14) The other substance was purified by ion exchange chromatography only since acid precipitation of the host antigen from the concentrated allantoic fluid gave an unsatisfactory yield (cf below under Experimental) The two preparations showed rather similar chemical composition

Ion exchange chromatography DEAE Sephadex columns for preparation of the host antigen were prepared and run as described before (14) DEAE cellulose (Serva grade p a) columns were prepared according [illegible] and were run by stepwise or gradient elution [illegible] Sephadex columns The host antigen was elut[illegible] at a lower NaCl molarity than from DEAE [illegible]

The authors are indebted to Mr A Bye Hansen Miss T Fotland and Mrs L Wang for skilled technical assistance

gentle as 0 04 N HCl at 100° C for 10 minutes The fucose spot showed maximal intensity after hydrolysis with 0 1 N HCl at 100° C for 20 to 30 minutes With stronger hydrolysis the fucose spot became weaker while the galactose spot became more intense The hexosamine spot appeared after hydrolysis with 0 1 N HCl at 100° C for 60 minutes together with a fourth spot with the same Rf value as N acetylglucosamine After treatment of a chromatogram with reagent 7 this new spot was stained blue typical of an N acetylhexosamine (which also was confirmed by examining the hydrolysate with the method of *Reissig et al* (22))

TABLE 1

Separation of Amino Sugars from an Acid Hydrolysate of the Host Antigen by Chromatography on Dowex 50 (11)

	Peak volume	β-glucosamine value†
Unknown amino sugar 1	68 1	0 98
Unknown amino sugar 2	81 9	1 18
Glucosamine standard	69 5	1 00
Galactosamine standard	85 3	1 23

The peak volume is the total effluent volume (ml 0 33 N HCl) at which an amino sugar is eluted at its maximum concentration (6)

† The β-glucosamine value is the peak volume of a substance relative to that of glucosamine (6)

Glucosamine galactosamine and mannosamine can usually not be distinguished by paper chromatography only Acid hydrolysates (4 N HCl at 100° C for 4 hours) were therefore chromatographed on 8 × 420 mm Dowex or Zerolit 225 columns and eluted with 0 33 N HCl for separation of the amino sugars The same column was also run with glucosamine and galactosamine standards Two amino sugars were recovered from the hydrolysate of the host antigen with elution volumes corresponding to glucosamine (or mannosamine) and galactosamine (cf Table 1) In order to differentiate between glucosamine and mannosamine which have nearly the same elution volumes (6) further examinations of their N acetylderivatives by paper chromatography and electrophoresis were necessary Another Dowex 50 column was run with hydrolysate of the host antigen as above and the unknown hexosamine taken to dryness by evaporation The dried material was dissolved in water and N acetylated with sodium bicarbonate and acetic acid anhydride as described by *Crumpton* (6) The sodium ions were thereafter removed by passing the material through a Dowex 50 column (6 × 160 mm) in the H form thoroughly pre washed with water The N acetylated amino sugar thus obtained was examined by paper chromatography in solvent system D (6) together with N acetylglucosamine and N acetylmannosamine reference substances The unknown N acetylhexosamine had the same Rf value as N acetylglucosamine but

significant separation of N-acetylmannosamine and N-acetylglucosamine was not obtained in this system —Paper electrophoresis was run with the same substances in a sodium tetraborate buffer of pH 10 The unknown N-acetylhexosamine had the same migration rate as N-acetylglucosamine, while N-acetylmannosamine moved faster with a migration rate relative to that of N-acetylglucosamine of about 1 8 The unknown amino sugar thus appeared to be glucosamine

Quantitative determination of glucosamine and galactosamine recovered from Zerolit 225 showed relative amounts of the two amino sugars of 2 1 1 and 1 9 1 in the two purified preparations respectively

As mentioned in the preceding article (14) gentle hydrolysis with 0 04 N HCl at 100° C did not split off the sulphate from the sugar, indicating that the sulphate was linked as an 0 ester and not attached to the amino groups of the hexosamine (10) This assumption has been further supported by the above mentioned demonstration of N-acetyl groups The main question was therefore whether the sulphate was bound to galactose or to the amino sugars A linkage to fucose was unlikely for several reasons Fucose was present in very low contents compared with the sulphate Fucose was released by gentle hydrolysis without having sulphate groups, which could not have been split off from the fucose by this mild hydrolysis Also part of the galactose was released by gentle hydrolysis without appearance of galactose sulphate The method described by *Rees* (21) was used in this search for sulphated monosaccharides By this method the migration rate of sulphated mono saccharides is greatly increased by the addition of cethyl pyridine chloride (CPC) to the solvent system As the sulphate obviously was linked to the amino sugar, one had to examine whether the acid hydrolysis conditions needed to release the amino sugar from the poly saccharide, also would split off the sulphate from the sugar A hydrolysis curve was therefore run, and the hydrolysates were examined by paper chromatography in solvent system B After hydrolysis with 0 1 N HCl at 100° C for 60 minutes, a very weak spot with the same Rf value as the reference N-acetylglucosamine 6-sulphate was detected with the silver nitrate reagent For further identification of this spot 30 mg of the host antigen was hydrolysed as above and applied to a DEAE cellulose column (9 × 150 mm) in the Cl form The column was then washed through with water and eluted with 0 5 M NaCl This eluate, containing possible sulphated sugars, was acetylated and lyophilized The sugars were extracted from this sugar-salt mixture by pyridine taken to dryness and extracted again twice The resulting material was examined in solvent system B, and the chromatogram stained with reagent 7 Three spots were obtained One near origo obviously representing oligosaccharide material, a second spot identified as N-acetylglucosamine, and a third fastmoving spot with an Rf value identical with our N-acetylglucosamine 6 sulphate reference Since the spot stained with the specific N-acetylhexosamine colour reagent (re-

gent 7), the presence of hexosamine ester sulphate in our antigen preparation was established. The sequence of release of the different sugars on acid hydrolysis has been summarized in Table 2.

TABLE 2

The Sequence of Release of Sugars on Acid Hydrolysis of the Host Antigen

Hydrolysis at 100° C with	Release of				
	Fucose	Galactose	Hexosamine	N-acetyl hexosamine	N-acetyl hexosamine sulphate
0.04 N HCl, 10 min	+	(+)	—	—	—
0.04 N HCl, 20 min	++	++	—	—	—
0.04 N HCl, 30 min	++	++	—	—	—
0.1 N HCl, 10 min	+	+	—	—	—
0.1 N HCl, 20 min	++	++	—	—	—
0.1 N HCl, 30 min	++	++	—	(+)	(+)
0.1 N HCl, 60 min	+	+++	+	+	+
4 N HCl, 4 hrs	trace	+++	+++	—	—

Code (+) to +++ denote intensity of spot on visual inspection of paper chromatograms.

Identification of the Amino Acids

Paper chromatography after strong acid hydrolysis (16 hours with 6 N HCl at 110° C) of the host antigen was carried out in solvent systems C, E and F. The following amino acids were identified: Aspartic acid, alanine, serine, threonine, proline, phenylalanine, valine and leucine (or isoleucine). The latter three amino acids gave very weak spots compared with the others. The identification of the amino acids was achieved by comparing the Rf values with standards, by the specific colour reagent for hydroxyamino acids (serine and threonine), by the typical staining of aspartic acid and proline with *Muting*'s reagent, and by two-dimensional chromatography in solvent systems E and C. Tryptophan and tyrosine could be excluded since the material showed no peak in the ultraviolet light region of 280 millimicrons (cf. below).

Infra red spectroscopy revealed absorption bands at 1653 and 1546 cm^{-1}, typical of acetamido groups. An intense band of 1250 cm^{-1} may be attributable to sulphate ester groups. The substance was too complex, however, for the study of more details in the region of 800 to 850 cm^{-1} with regard to the type of the sulphate linkage.

Free boundary electrophoresis revealed no signs of heterogeneity of the purified preparation. By descending boundary electrophoresis the mobility was 0.78×10^{-4} cm^2 sec volt, which corresponds well to a sulphated mucopolysaccharide (15).

Paper electrophoresis was carried out with 1.0 N acetic acid of pH 2.35 and with a 0.1 M barbitone acetate buffer of pH 8.6. Strips [illegible]

paper were cut parallel to the application line and eluted with water. The eluates were examined chemically and serologically without revealing any indication of heterogeneity of the material.

The ultraviolet spectrum of the purified material showed no peaks in the region between 210 and 300 millimicrons.

By polarimetry the material appeared to be weakly laevorotatory $[\alpha]_D^{13} = -17°$.

The chemical composition of the purified host antigen based upon the present chemical analyses is presented in Table 3. It appears that galactose and a presumed N-acetylhexosamine sulphate will account for about 75 per cent of the substance compared with a total recovery of 85 per cent. Correction for water uptake during hydrolysis has not been made.

TABLE 3

Chemical Composition of a Purified Preparation of the Host Antigen

	Content per cent of dry material	Molar ratios relative to SO_4	Method reference
Galactose	27	1.5	26
N-acetylglucosamine	24	0.9	2, 11
N-acetylgalactosamine	11	0.4	2, 11
Ester sulphate	12	1.0	8, 9
Fucose	4.3		7
Peptide (Folin)	5.8		16

DISCUSSION

It was reported in the foregoing article (14) that the purified host antigen behaved as a homogeneous material when passed through different Sephadex columns and in the analytical ultracentrifuge. The homogeneity of the material was further evidenced by the results of free boundary electrophoresis reported here, which also showed the strongly acidic properties of the antigen.

A total recovery of about 85 per cent of dry weight was obtained through chemical analyses. A complete release of hexosamines is, however, usually not possible on acid hydrolysis and, as pointed out in the foregoing article (14), the *Folin* value for the peptide content is probably too low since the substance did not contain tyrosine and tryptophan.

The chemical analyses showed about equimolar amounts of galactose and hexosamine. As might be expected the fucose was readily split off by acid hydrolysis in addition to a small part of the galactose. It is reasonable to assume a backbone structure of N-acetylhexosamine sulphate and galactose with fucose (and galactose?) end groups and a small peptide moiety. Aside from the ester sulphate groups this basic

structure is much like that of the human blood group substances A B and H (17)

So far it has not been possible to prepare greater amounts of the hexosamine sulphate to exclude that it originates from a minor im purity It also remains to be established whether the sulphate is bound to the glucosamine alone or to both amino sugars and to define the *position of the sulphate linkage in the hexosamine Other methods for* recovering the hexosamine sulphate are needed to get sufficient quan tities for these studies

It seems likely that the sulphate groups of the host antigen are re sponsible for the binding of this antigen to the haemagglutinin of allantoically cultured influenza virus The effects exerted on virus particles by polyelectrolytes have recently been reviewed by *Voss* (25) The present investigation seems to demonstrate another effect the lending to the virus of an antigen characteristic of the host

SUMMARY

The purified mucopolysaccharide was found to be homogeneous when *examined by free boundary and paper electrophoresis Paper chromato* graphy and ion exchange chromatography of acid hydrolysates revealed the sugars galactose, glucosamine galactosamine and fucose The amino acids were aspartic acid, alanine serine, threonine, proline and in trace amounts phenylalanine valine and leucine (or isoleucine) On gentle hydrolysis the amino sugars were released as the N acetyl derivatives and a small amount of N acetylhexosamine sulphate was also demon strated

Galactose and the presumed N acetylhexosamine sulphate appeared to be the major components of the polysaccharide accounting for about 75 per cent of the material The fucose content was 4 per cent and the peptide 6 per cent (Folin)

REFERENCES

1 *Block R J Durrum E L & Zweig G* A manual of paper chromatography and paper electrophoresis p 137 2nd edition Academic Press Inc New York 1958

2 *Boas N F* Method for the determination of hexosamines in tissues J Biol Chem 204 553 563 1953

3 *Bull H B Hahn J W & Baptist V H* Filter paper chromatography J Am Chem Soc 71 550–553 1949

4 *Cifonelli J A & Smith F* Detection of glycosides and other carbohydrate com pounds on paper chromatograms Anal Chem 26 1132 1134 1954

5 *Consden R Gordon A H & Martin A J P* Qualitative analysis of proteins A partition chromatographic method using paper Biochem J 38 224–232 1944

6 *Crumpton M J* Identification of amino sugars Biochem J 72 479 486 1959

7 *Dische Z* New color reactions for the determination of sugars in polysacch arides pp 313 358 Methods of biochemical analysis vol II Interscience Publishers Inc New York 1955

8 *Dodgson K S & Price R G* A note on the determination of the ester sulphate content of sulphated polysaccharides Biochem. J 84 106 110 1962

9 *Dodgson K S & Spencer B* Studies on sulphatases 5 The determination of inorganic sulphate in the study of sulphatases Biochem J 55 436 440 1953

10 *Foster A B Martlew F F Stacey M Taylor P J M & Webber, J M* Amino sugars and related compounds Part VIII Some properties of 2 deoxy 2-sulphoamino D glucose heparin and related substances J Chem Soc 1204 1208, 1961

11 *Gardell S* Separation on Dowex 50 ion exchange resin of glucosamine and galactosamine and their quantitative determination Acta chem scandinav 7 207 215 1953

12 *Harboe A* The influenza virus haemagglutination inhibition by antibody to host material Acta path et microbiol scandinav 57 317–330 1963

13 *Harboe A* The normal allantoic antigen which neutralizes the influenza virus HI antibody to host material Acta path et microbiol scandinav 57 488–492 1963

14 *Haukenes G Harboe A & Mortensson Egnund K* A uronic and sialic acid free chick allantoic mucopolysaccharide sulphate which combines with influenza virus HI antibody to host material 1 Purification of the substance Acta path et microbiol scandinav 64 534 542 1965

15 *Larsen B* The Norwegian Institute of Seaweed Research Trondheim Personal communication 1965

16 *Lowry O H Rosebrough N J Farr A L & Randall R J* Protein measurement with the Folin phenol reagent J Biol Chem 193 265–275 1951

17 *Morgan W T J* Mucopolysaccharides associated with blood group specificity Ciba Foundation Symposium on the Chemistry and Biology of Mucopolysaccharides pp 209–211 J & A Churchill Ltd London 1958

18 *Müting D* Über eine qualitative Farbreaktion zur Differenzierung von Aminosäuren auf Filtrierpapier Naturwissenschaften 39 303 1952

19 *Pallavicini J C Gabriel O di Sant Agnese P A Buskirk F R* Isolation and characterization of carbohydrate protein complexes from human sweat N Y Acad Sci 106 330–338 1963

20 *Partridge S M* Filter paper partition chromatography of sugars 1 General description and application to the qualitative analysis of sugar in apple juice egg white and foetal blood of sheep Biochem J 42 238 250 1948

21 *Rees D A* Paper chromatography of acidic carbohydrates Nature 185 309 310 1960

22 *Reissig J L Strominger J L & Leloir L F* A modified colorimetric method for the estimation of N acetylamino sugars J Biol Chem 217 959 966 1955

23 *Suzuki S & Strominger J L* Isolation and identification of acetylgalactosamine monosulfates J Biol Chem 235 2768–2773 1960

24 *Trevelyan W F Procter D P & Harrison J S* Detection of sugars on paper chromatograms Nature 166 444–445 1950

25 *Voss H* Virus and Polyelektrolyte II Mitteilung Die Bedeutung der Polyelektrolyte für die Abwehr antigenwirksamer Substanzen und Erreger im Organismus Zbl Bakt., I O 103 1 33 1964

26 *Whitehouse M W & Bostrom H* Studies on the action of some anti inflammatory agents in inhibiting the biosynthesis of mucopolysaccharide sulphates Biochem Pharmacol 7 135 150 1961

27 *Winzler R J* Determination of serum glycoproteins pp 279 311 Methods of biochemical analysis vol II Interscience Publishers Inc New York 1955

Acta path et microbiol scandinav 66 519 529 1966

Virus Department A National Institute of Public Health Oslo Norway

STUDY OF THE CELL BOUND INHIBITOR OF POLYOMA VIRUS HAEMAGGLUTINATION

By

DORIS DAHL, JON JONSEN and O LAHELLE

Received 20 viii 65

The haemagglutinating activity of polyoma virus can be inhibited by substances from various sources The inhibition can be demonstrated with medium or cells from cultures infected with polyoma virus (*Hartley & Rowe* 1959 *Hartley et al* 1959) but inhibitors have repeatedly also been obtained from normal cells Thus, homogenates of mouse and hamster tissue (*Diderholm & Wesslen* 1963), organ extracts from normal and immune mice (*Balduzzi & Salmon* 1962), homogenates of secondary mouse cells grown in tissue culture (*Deinhardt et al* 1960) and extracts of mouse cells and HeLa cells (*Salmon & Berdondini* 1964) were all found to inhibit haemagglutination

The inhibitory activity of culture fluid from polyoma infected tissues and of bovine serum decreased markedly by treatment with receptor destroying enzyme trypsin and fluorocarbon the persistence of some inhibiting activity suggesting however the presence of at least two different inhibitors (*Deinhardt et al* 1960 *Halperen et al* 1962 *Hartley & Rowe* 1959)

Mori et al (1961) reported the presence of five heat stable proteins with inhibitory activity from bovine serum One of these had properties similar to those of the erythrocyte receptors another was proposed to be analogous to a host cell receptor (*Mori et al* 1961 1962) *Holland* (1961) established that receptor affinities were major determinants of enterovirus tissue tropism The receptor probably a lipoprotein was located in the cell membranes and mainly found in the microsome fraction of the susceptible HeLa cells but not in insusceptible cells like L strain mouse fibroblasts (*Holland & McLaren* 1961) With polyoma virus Crawford (1962) however observed unspecific adsorption to both susceptible cells and to insusceptible HeLa cells

The present study compares the adsorption of polyoma virus to susceptible and non susceptible cells grown in suspension culture The haemagglutinin inhibition of whole cells cell homogenates and subcellular particles has been tested Some properties of the cell bound inhibitor from susceptible and non susceptible cells have been compared

MATERIALS AND METHODS

Cell cultures Primary cultures of mouse embryo cells were grown in Eagle's minimum essential medium (*Eagle* 1959) with Hanks balanced salt solution and a supplement of 20 per cent calf serum After infection the medium was changed to Eagle Hanks medium with 2 per cent horse serum

P 388 D1 cells[1] and Detroit 6 cells[2] were maintained as monolayer cultures in Roux bottles and transferred to suspension flasks when grown for experiments For monolayers the cells were grown in Eagle's medium with 5 per cent calf serum 10 per cent serum was used when the cells were grown in suspension culture

Virus A subline of SE polyoma virus originally obtained from Dr Herbert Morgan University of Rochester was used

Virus production Virus was cultured in primary mouse embryo cells grown in Roux bottles The cells were inoculated with an input multiplicity of infection of 2 3 plaque forming units (PFU) per cell using infectious tissue culture fluid clarified by low speed centrifugation After incubation at 37° C for 8–10 days the cells were scraped into the medium harvested by centrifugation at 2000 rpm for 10 minutes at 4° C and washed three times with phosphate buffered saline (*Dulbecco & Vogt* 1954) at pH 7 2 The cells were then suspended in phosphate buffered saline (PBS) disrupted by freezing and thawing four times and 1 part of the receptor destroying enzyme of Vibrio cholerae prepared according to the directions of the supplier was added to 10 parts of cell debris (*Crawford* 1962) After incubation at 37° C for 20 hours followed by heating to 56° C for 30 minutes the cell debris was sedimented at 4° C by centrifugation at 9000 g for 10 minutes The supernatant containing the virus was stored at —20° C

Preparation of cell suspensions and homogenates The cells were scraped off the glass surface harvested by centrifugation at 2000 rpm for 10 minutes at 4° C washed three times with PBS (4° C) and resuspended to 10^7 cells/ml The cell suspension was then treated in a Potter Elvehjem grinder at 4° C and the disruption of the cells were controlled by counting intact cells in the preparation

Fractionation of cells Subcellular fractions of P 388 D1 cells and Detroit 6 cells were prepared according to the method described by *Holland & McLaren* (1961) The nuclear fraction was sedimented at 800 g for 100 minutes the mitochondrial fraction at 7000 g for 10 minutes the microsomal fraction at 38 000 g for 30 minutes and the postmicrosomal fraction at 105 000 g for 2 hours The nuclear fraction was washed twice the mitochondrial microsomal and postmicrosomal fractions were washed once in 0 25 M sucrose All fractions were resuspended in PBS to 2 10^7 cells/ml before testing for inhibitory activity

Contamination of the nuclear fraction with whole cells was less than 3 per cent and contamination of the mitochondrial fraction by cell fragments or nuclei was less than 2 per cent

RESULTS

The Adsorption of Polyoma Virus Haemagglutinin to Susceptible and Non Susceptible Tissue Cultures Cells

P 388 D1 cells were chosen as the host cell type Detroit 6 cells as the non susceptible cell type The adsorption experiment was performed as follows Cells were grown in suspension cultures harvested by centrifugation washed three times with PBS and resuspended in PBS 5 $10^{[illegible]}$ cells were suspended in 0 8 ml PBS and 1024 HA units of virus in 0 [illegible] ml PBS were added The samples were incubated at 4° C 22° C and 37° C for intervals up to 90 minutes and the adsorption was interrupted by sedimenting the cells at the incubation temperature (4000 rpm [illegible] minutes) The supernatant was treated with an equal volume of RDE

---

[1] Obtained from Dr Robert A Roosa Wistar Institute Philadelphia

[2] Purchased from Microbiological Associates Inc Bethesda

for 20 hours at 37° C and then tested for virus haemagglutinins ('free virus") The sediment was washed once with 1 ml PBS at 4° C and then incubated with 0 5 ml RDE and 0 5 ml PBS at 37° C for 20 hours followed by inactivation of the enzyme by heating to 56° C for 30 minutes The suspension was centrifuged as before The HA titre of the supernatant was defined as a measure of adsorbed virus A second treatment with RDE did not release significant amounts of haemagglutinins Following the second RDE incubation, the sediment was lysed by incubation at 22° C for 10 minutes in the presence of 0 2 per cent sodiumdodecylsulphate (SDS) as described by *Mandel* (1962) No intracellular haemagglutinins could be detected by this method The adsorption of viral haemagglutinins at 4° C seemed to be slightly higher than the adsorption at 37° C and probably higher than the 22° C adsorption also (Table 1) No significant difference between the adsorption to suspectible and non susceptible cells was observed The ability of 'free virus" to readsorb was not tested

TABLE 1

Adsorption of Polyoma Virus Haemagglutinins to P 388 D1 Cells and Detroit 6 Cells at 4° C 22° C and 37° C

Temperature	Minutes adsorption	HA titre of P 388 D1 assay		HA titre of Detroit 6 assay	
		Absorbed virus†	Free virus	Absorbed virus	"Free virus
4° C	0*	80	640	80	640
	30	160	320	320	320
	60	320	320	320	320
	90	320	160	320	320
22° C	0	80	640	80	640
	30	80	320	160	320
	60	160	320	160	160
	90	160	320	320	160
37° C	0	80	640	160	640
	30	80	640	160	640
	60	160	320	80	640
	90	160	320	160	320

* Samples centrifuged immediately after addition of the virus Total amount of haemagglutinins added was 1024 HA units

§ Virus sedimenting with the cells and eluted by RDE incubation of the washed sediment

† Virus remaining in the supernatant after centrifugation (4000 rpm 5 min)

Inhibition of Polyoma Virus Haemagglutination by Susceptible and Non Susceptible Tissue Cultures Cells

been cultivated in Roux bottles and the monolayers were scraped off HeLa cells, Detroit 6 and P 388 D1 cells gave the same inhibition titres while mouse embryo cells possibly had a lower activity (Table 2) The HAI titre increased on disruption of the cells to cell homogenates The increase in titre was usually twofold, sometimes a 4-fold increase was observed

TABLE 2

Inhibition of Polyoma Virus Haemagglutination by Intact Cells and Homogenates from Different Cell Types

Test material*	HAI†
Mouse embryo cells	1024
Homogenized mouse embryo cells	2048
P 388 D1 cells	2048
Homogenized P 388 D1 cells	4096
Detroit 6 cells	4096
HeLa cells	2048
Homogenized HeLa cells	4096

* Concentrated suspensions of 10 cells/ml
† Standard HAI assay

Location of Polyoma Virus Inhibitor in Subcellular Fractions of P 388 D1 and Detroit 6 cells

The mitochondrial fraction inhibited haemagglutination to a higher degree than the other particle fractions and a substantinal amount of inhibitor was found in the cell sap (Table 3 and 4) In some experiments the freshly isolated nuclei gave a low inhibitory titre which increased after disrupting the nuclei by freezing and thawing Additional cycles of freezing and thawing resulted in a decrease in inhibitory titre as it did for all subcellular fractions (Table 5) No marked differences in inhibitor distribution were observed between the two cell types examined

TABLE 3

Inhibition of Haemagglutination by Subcellular Fractions from P 388 D1 Cells

Cell fraction*	Haemagglutination—inhibition titres†			
	2 hours	4 hours	6 hours	20 hours
Intact cells	128	256	512	1024
Cell homogenate	256	512	1024	4096
Nuclei	8	32	64	128
Mitochondria	32	64	256	512
Microsomes	16	32	64	128
Postmicrosomes	8	16	32	64
Cell sap	64	64	128	256

* Referring to a cell concentration of 2 10 cells/ml of subcellular fractions from 2 10 cells/ml
† Dilution series of the subcellular fractions were incubated with 8 HA units of virus at 4° C for the time indicated before the addition of erythrocytes

TABLE 4

Inhibition of Haemagglutination by Subcellular Fractions from Detroit 6 Cells

Cell fraction*	Haemagglutination—inhibition titres†			
	2 hours	4 hours	6 hours	20 hours
Intact cells	128	256	512	1024
Cell homogenate	256	512	1024	4096
Nuclei	16	32	64	128
Mitochondria	64	128	512	1024
Microsomes	32	64	128	256
Postmicrosomes	16	32	64	128
Cell sap	128	256	256	1024

* Referring to a cell concentration of 2 10 cells ml of subcellular fractions from 2 10 cells ml

† Dilution series of the subcellular fractions were incubated with 8 HA units of virus at 4° C for the time indicated before the addition of erythrocytes

TABLE 5

Decrease of Inhibitory Activity in Subcellular Fractions of Detroit 6 Cells by Freezing and Thawing

Cell fraction	Haemagglutination—inhibition titres*			
	Freshly prepared	Frozen thawed once	Frozen thawed twice	Frozen thawed three times
Nuclei	128	128	256	32
Mitochondria	1024	256	256	64
Microsomes	256	64	64	32
Postmicrosomes	128	64	64	32
Cell sap	1024	256	256	128

* Standard HAI assay

Time Course and Temperature Dependence of the Interaction between Polyoma Virus Haemagglutinins and Inhibitor of P 388 D1 Cell Sap

The rate of virus inhibitor interaction was determined by adding red blood cells to virus cell sap mixtures which had been incubated for varying periods of time The experiment showed (Table 6) that the interaction pattern at 4° C and at 22° C was very similar The reaction between virus and inhibitor at 37° C was less extensive The maximum titres were much lower and a decline of virus titre at the low dilutions used in the HAI titration made the study of the interaction difficult for longer time periods Preincubation of the inhibitory material with erythrocytes before the addition of virus had no effect on the HAI titres showing that the inhibition acts on the virus independently of the presence of erythrocytes

The same results were obtained with *P 388 D1 cell homogenates*

Interaction of subcellular fractions of P 388 D1 cells with virus at

37° C for 1 hour gave lower HAI titres for all fractions when compared with the titres obtained after interaction at 4° C for 2 hours Interaction for 20 hours at 4° C was adapted for the standard HAI titration

TABLE 6
The Temperature Dependence of the Interaction between Polyoma Virus and P 388 D1 Cell Sap

Time*	Haemagglutination—inhibition titres†		
	4° C	22° C	37° C
5 minutes	8	16	8
15 "	16	32	16
30 "	32	64	16
45 "	64	128	16
60	128	128	64
2 hours	256	128	8
3	512	256	4
4	256	128	not done
5 "	1024	256	"
6	1024	1024	"
16	1024	1024	"
20 "	1024	1024	"

* Time of incubation of virus inhibitor at the temperature indicated before the addition of erythrocytes
† HAI titres referring to the cell sap obtained from 10^7 cells ml

Chemical Characteristics of the Cell Bound Inhibitor

The sensitivity of the inhibiting effect of cell homogenates to various treatments was investigated Cell homogenates from mouse embryo cells, P 388 D1 cells and Detroit-6 cells were tested

Dialysis Five ml of homogenates (5 10^6 cells/ml) were dialyzed against 500 volumes of PBS at 4° C for 24 hours The PBS was changed once

Heat The homogenates (2 10^6 cells/ml) were heated in a water bath for various periods of time at 56° C and 100° C followed by cooling in an ice-bath

Treatment with enzymes The cell homogenates contained 5 10^6 cells/ml in all enzyme assays The following tests were made

Receptor destroying enzyme 1 part cell homogenate and 1 part RDE were incubated at 37° C for 30 minutes The enzyme was inactivated by heating at 56° C for 30 minutes before HAI titration

Trypsin and chymotrypsin were added to heated (100° C for 30 minutes) and unheated samples of homogenate to a final concentration of 10 mg ml and the mixtures incubated for 20 hours at 37° C

Papain at a final concentration of 10 mg ml was incubated with the cell homogenates at 56° C for 1 hour The buffer for this enzyme assay was a 0 1 M phosphate buffer, pH 6 5, containing 0 005 M EDTA and 0 005 cysteine The enzyme was preheated in the buffer at 65° C for 30 minutes

Ficin in the same concentration and in the same buffer as described for papain was incubated with the cell homogenates at 37° C for 20 hours

Pronase, 0 5 mg ml and 1 0 mg ml in 0 2 M Tris buffer, pH 8, was incubated with the cell homogenate at 37° C for 20 hours

Enzymes Receptor destroying enzyme of Vibrio cholerae was obtained from Philips Duphar, Amsterdam Neuraminidase from Clostridium perfringens was the purified type 5 from Sigma Chemical Company

Houses Ltd and ficin from Sigma Chemical Company Pronase from Streptomyces griseus was obtained from Kaken Kagaku Co Ltd Tokyo

Haemagglutination (HA) titrations The assays were made by adding 0 2 ml of a 0 4 per cent suspension of guinea pig red cells to 0 2 ml of virus diluted in PBS The erythrocytes were suspended in PBS containing 0 9 per cent bovine serum albumin Readings were made after incubation at 4° C for 3 hours One HA unit was the amount of virus required to give partial agglutination in this system

Haemagglutination inhibition (HAI) titrations Serial twofold dilutions of 0 2 ml samples were tested for inhibitory activity by incubation for 20 hours at 4° C with 8 haemagglutinating units of virus in 0 2 ml Subsequently 0 4 ml of a 0 4 per cent suspension of guinea pig red cells were added (4° C) and the HAI titre was read after 2 hours at 4° C. One HAI unit was the amount of inhibitor required to give partial haemagglutination inhibition in this assay system

Organic solvents The cell homogenates were treated with different organic solvents and HAI titres determined in the water phases

The following procedures were used

1 One part of cell homogenate 5 10^6 cells/ml) was emulsified in an omnimixer with one part of a fluorocarbon Genetron for 6 minutes at 4° C and 22° C The water phase was collected following centrifugation at 400 rpm for 10 minutes 4° C.
2 One part of cell homogenate (5 10^6 cells/ml) was shaken for 15 minutes with two parts of butanol-chloroform (1 5) at 4° C and 22° C The water phase was collected as above
3 Treatment with SDS 0 2 per cent was carried out at 22° C for 20 minutes The SDS reaction mixtures lysed the erythrocytes in dilutions lower than 1 8 but this had no practical importance for the HAI titration as the inhibitory titre decreased only slightly by the treatment
4 *Periodate* The inhibitor was treated with varying (0 003 M 0 01 M) concentrations of potassium periodate at 37° C for 30 minutes The periodate action was stopped by cooling and by the addition of one volume 10 per cent glucose

Table 7 shows that RDE as well as treatment with periodate completely destroyed the inhibitory activity The proteolytic enzymes papain, ficin and pronase also had a pronounced effect on the inhibitor whereas trypsin and chymotrypsin had no effect

The deproteinizing agents fluorocarbon and butanol chloroform decreased some of the inhibitory activity the lipid solvents, SDS and ether, had a rather slight effect on the inhibitor The inhibitor seemed to be heat resistant and the activity was not affected by dialysis The slight decrease of the HAI titre after heating at 100° C could be due to coprecipitation of the inhibitor with the other proteins in the homogenate

TABLE 7

The Sensitivity of the Haemagglutination Inhibitory Activity from Cell Homogenates to Various Treatments

Treatment	HAI titre of homogenates from					
	P 388 D1 cells		Mouse embryo cells		Detroit 6 cells	
	Before	After	Before	After	Before	After
Dialysis	256	256	128	128	512	512
Heat						
56° C 30 min	256	256	128	128	1024	512
100° C 30 min	256	128	128	64	512	256
RDE	256	0	128	4	512	0
Trypsin						
Heated homogenate	128	128	64	64	256	256
Unheated homogenate	256	256	128	128	512	512
Chymotrypsin						
Heated homogenate	512	512	256	256	1024	1024
Unheated homogenate	512	512	512	512	2048	2048
Papain	1024	32	256	8	512	0
Ficin	1024	8	512	16	1024	0
Pronase						
1 0 mg ml	2048	512	128	32	1024	64
Fluorocarbon						
4° C 6 min		–	–	–	512	16
22° C 6 min		–	–	–	512	32
Butanol chloroform						
4° C 15 min	–	–			512	16
22° C 15 min	–	–	–		512	32
SDS	256	128	128	64	512	512
Ether	256	128	128	64	512	128
KIO_4						
37° C 30 min 0 01 M	1024	0	512	0	1024	16

TABLE 8

The Sensitivity of the Haemagglutination Inhibitory Activity in Cell Sap from P 388 D1 Cells to Various Treatments

Treatment	HAI titre of P 388 D1 cell sap	
	Before	After
Heat		
100° C 30 min	1024	512
RDE from Vibrio cholerae	1024	0
Neuraminidase	1024	16
Trypsin		
Heated cell sap	256	256
Unheated cell sap	512	512
Chymotrypsin		
Heated cell sap	256	256
Unheated cell sap	512	512
Papain	256	0
Ficin	1024	256
Pronase	1024	0

TABLF 9

Effect of the Cell Bound Inhibitor on the Infectivity of Polyoma Virus

Temperature of interaction	PFU 10^c following preincubation with		
	Phosphate buffered saline	Mouse embryo cell homogenate	Detroit 6 cell homogenate
4° C	3 7	3 9	4 1
2? C	4 0	4 4	4 5

Average of four plates

The cell sap from P 388 D1 cells was treated with some of the above agents No pronounced differences could be observed on the effect of these agents on the inhibitor of the cell sap and the inhibitor substance in the cell homogenate (Table 8) When adding TCA to the cell sap to a final concentrations of 2 5 per cent most of the inhibitory activity remained in the supernatant

The Effect of Inhibitor on Virus Infectivity

Cell homogenates (10 cells ml) of mouse embryo cells and Detroit 6 cells were used in these assays The infectivity was tested (Table 9) after incubation of 2 ml cell homogenate with 10 plaque forming units at 4° C and 22° C

DISCUSSION

Suspensions of whole cells and of homogenates of cultured cells were found to inhibit the haemagglutination caused by SE polyoma virus HeLa cells and Detroit 6 cells which are insusceptible to infection by polyoma virus showed a higher haemagglutination inhibition than the susceptible mouse embryo cells The same observation was recently reported by *Salmon & Berdondini* (1964) using extracts from HeLa cells and secondary cultures of mouse embryo cells The susceptible cell line P 388 D1 and the insusceptible Detroit 6 cells showed the same degree of inhibition in most preparations

Disrupting cells by homogenization increased the inhibitory capacity of all the cell types tested in agreement with experiments made on enteroviruses (*Holland & McLaren* 1961 *Philipson & Bengtsson* 1962) and polyoma virus (*Diderholm & Wesslen* 1963) All the subcellular particulate fractions of P 388 D1 and Detroit 6 cells contained inhibitor with the nuclear fraction having the lowest and the mitochondrial fraction the highest inhibitory capacity The inhibition of the nuclear fraction increased by breaking up the nuclei in the same way as was found by disruption of the intact cell In our experiments the cell sap also showed a high inhibiting capacity in agreement with the findings of *Diderholm & Wesslén* (1963) The inhibiting activity of the micro

some fraction was rather low, however Probably the distribution of inhibitory material may vary with the cell type studied, but it seems rather certain that inhibitory material is located in all parts of the cell, a situation very much different from the limited distribution of type 1 poliovirus receptors in HeLa cell membranes (*Holland & McLaren* 1961) *Philipson & Bengtsson* (1962) reported the microsome fraction to be the most effective in inhibiting both the haemagglutination of the haemagglutinating enteroviruses and the infectivity of Echo 7 and poliovirus type 1, but they found also significant inhibitory activity present in the cell sap and the postmicrosome fraction although the inhibitor in the cell sap had little effect on the poliovirus infectivity

Treatment of the cell bound inhibitor with different agents indicated that the inhibitor is a mucoprotein like the mucoprotein receptors to polyoma virus in red blood cells as described by *Hartley et al* (1959), *Mori et al* (1962) and also like the inhibitor isolated from bovine serum by *Halperen et al* (1962) The inhibitor activity could be completely destroyed by the action of RDE and periodate and by incubation with proteolytic enzymes like papain, ficin and pronase Trypsin and chymotrypsin had no effect on the inhibitor. The heat stability of the inhibitor and the solubility of the inhibitor of the cell sap in 2 5 per cent TCA also indicated a mucoprotein structure The partial destruction by fluorocarbon and butanol-chloroform might be due to the deproteinizing effect of these solvents, the resistance to ether and detergent indicating that no lipid structures are involved in the inhibition

With the chemical treatment here described no difference could be observed between inhibitors from host cells like mouse embryo cells and P 388 D1 cells when compared with inhibitor from the non-sensitive cell type, Detroit-6 cells

Nor could the inhibitor from the cell sap of P 388 D1 cells be distinguished from particle bound inhibitor present in the cell homogenate The complete destruction of inhibitor by neuraminidase and periodate and the resistance to trypsin distinguished the cell bound inhibitor from the inhibitor isolated by *Halperen et al* (1962) from bovine serum and which was proposed to be identical with the reactive component of the red cell surface The cell bound inhibitor in our experiments which reacted to a similar extent at 4° C and 22° C and the lack of effect on the infectivity at these temperatures also seemed different from the bovine serum inhibitor which reacted at 22° C and blocked both the infectivity and the haemagglutination of polyoma virus (*Mori et al* 1961)

SUMMARY

Haemagglutination inhibition of polyoma virus by whole cells and cell homogenates of susceptible and non-susceptible cells was studied No correlation between inhibiting effect and susceptibility to polyoma virus infection was observed The disrupted cells showed higher inhibition

than intact cells demonstrating the presence of intracellularly located inhibitory substances The cell bound inhibitor was distributed in all subcellular fractions the highest inhibitor concentrations being found in the mitochondrial fraction and in the cell sap The inhibition by the nuclear fraction was relatively low but increased by disruption of the nuclei The inhibitor virus interaction took place to a similar extent at 4° C and 22° C the interaction at 37° C was low The chemical properties of the inhibitor were different from those described for inhibitors of bovine serum No differences could be observed in the properties of the soluble inhibitor in the cell sap and the particle bound inhibitor nor between the inhibitors of susceptible and non susceptible cells

REFERENCES

Ballu z P & Salmon
s eri e organi d
Crawford I V The a
De nhardt F Henle G
ing activity of po
D derholm H & Wesslen T Interaction between polyoma virus and different tissues of mouse and hamster *in vitro* Archiv f Virusforschung *14* 45–54 1963
Dulbecco R & Vogt M Plaque formation and isolation of pure lines with polyomyel tis virus J Exp Med *99* 167 182 1954
Eagle H Amino acid metabolism n mammalian cell cultures Science *130* 432 437 1959
Halperen S Schieble J H Mori R Dinka S & Ackermann W W Some propert es of an inhibitor of polyoma virus hemagglutination isolated from bovine serum J Immunol *89* 400–407 1962
Hartley J W & Rowe W P "Unmask ng of mouse polyoma virus hemagglutin n b heat and RDE Virology 7 249 250 1959
Hartley J W Rowe W P Chanock R M & Andrews B F Stud es of mouse p ly oma virus infecti n 4 Evidence for mucoprotein erythrocyte receptors in polyoma virus hemagglutination J Exp Med *110* 81 91 1959
Helgeland K., Jonsen J & Lahelle O Polyoma virus 2 Further stud es on the interaction of polyoma virus with mouse embryo cells at low temperatures Acta path et microbiol scandinav *63* 79 90 1965
Holland J J Receptor affinities as major determinants of enterovirus t ssue tropisms in humans Virology *15* 312 326 1961
Holland J J & McLaren L The location and nature of enter v rus receptors in susceptible cells J Exp Med *114* 161 171 1961
Mandel B The use of sod um dodecyl sulfate in stud es on the interaction of p l v rus and HeLa cells Virology *1* 288 294 1962
Mori R Sch eble J H & Ackermann W W Reacti n of poly ma and influenza viruses with receptors of erythrocytes and h st-cells Proc Soc Exp B ol Med *109* 685 690 1962
Mori R Schieble J H D nka S & Ackermann W W Serum inh b t rs of p lyoma virus Bact Proc 158 1961
Ph l pson L & Bengtsson S Interaction of enter viruses with receptors fr m erythrocytes and host cells Virology *18* 457–469 1962
Salmon S & Berdond ni I Inh bitori della emoagglutinaz one da v rus p l oma in animali di specie d verse e in cellule e ltivate Lo Sperimentale *114* 56 6 1964

Acta path et microbiol scandinav 66 530 540 1966

Department of Tumor Biology, Karolinska Institutet Stockholm 60 Sweden

STUDIES AT THE CELLULAR LEVEL OF THE 19S IMMUNE RESPONSE

By

HANS WIGZELL, GORAN MÖLLER and BIRGER ANDERSSON

Received 8 x 65

The relative rate of antibody synthesis increases with the antigen dose up to an optimal dose level with a minimal doubling time of the 19S serum antibody titres of approximately 6 hours (*Uhr & Finkelstein* 1963, *Svehag & Mandel* 1964) This relationship between antigen dose and rate of antibody production during the exponential phase of antibody synthesis has tentatively been attributed to proliferation of antibody producing cells and/or an exponential increase in the absolute rate of antibody synthesis per cell (*Bradley & Watson* 1963, *Urso & Makinodan* 1963, *Uhr & Finkelstein* 1963, *Svehag & Mandel* 1964)

In order to study these problems in a more detailed way we have in the present article been using a delicate method detecting single antibody forming cell *in vitro* (*Jerne & Nordin* 1963) Particular interest has been attached to the effect of antigen dose on the number of antibody-producing cells as compared to the serum titres during the early exponential phase of antibody synthesis

MATERIAL AND METHODS

Mice of the inbred strains CBA C3H A/Sn A SW A Ca A BY C57BL/10 and C57L and their F_1 hybrids have been used Unless otherwise stated in the text the animals were 2 3 months of age and had a body weight of about 20 g The strains were maintained by strict brother sister mating All animals in each experimental group were of the same strain sex and age

The haemolytic plaque test was carried out according to *Jerne & Nordin* (1963) Cell suspensions assigned for the estimation of plaque forming cells (PFC) were obtaining by pressing the organs through a 60 mesh stainless steel screen into Eagle s medium in Earle s solution without glutamic acid (*Eagle* 1955) The viability of the cells was determined by supravital staining with trypan blue and subsequent counting in a haemocytometer

Haemagglutination was carried out using the PVP method of *Stimpfling* (1961) Haemolysis was performed by serially diluting 0 025 ml of antiserum in a phosphate buffer (see gel filtration) and subsequently adding 0 025 ml of a 2 per cent sheep

---

We are grateful to Prof *Georg Klein* for his valuable support and advice and we thank Miss *Gertrud Vejlens* and Miss *Lena Lundin* for their elegant technical assistance This work was supported by grants from the *Swedish Cancer Society* the *Swedish Medical Research Council* and by grant CA–03700 from the *National Cancer Institute U S Public Health Service*

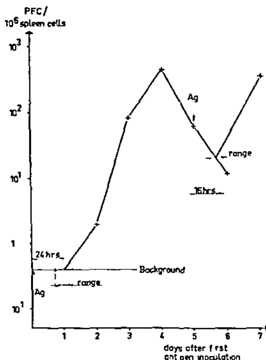


Fig 1

Lag period for PFC to increase after a primary challenge or rechallenge with 4×10^8 sheep erythrocytes Each dot represent the geometric means of values obtained from twenty animals

red cell suspension and 0 025 ml of fresh guinea pig serum diluted 1 20 as a source of complement Incubation was at 37° C for one hour The highest antibody dilution showing 100 per cent lysis was taken as the endpoint The indirect fluorescent anti body test was carried out according to *Moller* (1961) using a rabbit anti mouse globulin serum which had been fluorescein conjugated and shown by immunoelec trophoresis to contain antibodies against both 19S and 7S mouse gamma globulin The endpoint in this test was taken as 50 per cent fluorescing cells Inactivation of the antibodies with 2 mercaptoethanol was carried out according to *Uhr & Finkel stein* (1963)

Gel filtration of the antisera was performed with Sephadex G200 0 2 0 5 ml of serum was placed on the top of a 2×45 cm column and the separation was carried out at room temperature in M/15 phosphate buffer pH 7 4 containing 0 14 g of

storage The 19S and 7S fractions respectively were pooled and concentrated by ultrafiltration against PVP by negative pressure dialysis or by mixing the protein fractions with Sephadex G 25

RESULTS

As a rule normal mouse spleens contain a certain number of "spon taneously occuring PFC (*Jerne & Nordin* 1963, *Sterzl & Mandel* 1964) The time needed for the PFC to increase above the background level was now studied using an optimal dose of sheep red cells, 4×10^8 Four

experimental and four control animals were tested every four hours after inoculation Fig 1 shows the mean values of ten different experiments in order to establish the lag period after a primary and secondary antigen inoculation The primary lag period was 24 hours (range 14–32 hours) and the secondary response was detectable after 16 hours (range 12–18 hours

TABLE 1

Effect of Antigen Dose on the Rate of Increase of PFC and on the Peak Day of the Maximum Number of PFC

Strain	No of sheep red cells	Doubling time of PFC in hours	Peak day of PFC	Maximum number of *PFC/10⁶ cells**
A SW †	4×10^6	16.8	6	8.5
	4×10^8	10.1	5	44.0
A SW × C57BLF$_1$	4×10	14.5	6	18.0
"	4×10^7	11.2	6	41.0
	4×10^8	6.4	5	245.0
"	4×10^9	6.0	4	410.0
A SW × CBAF$_1$	4×10^6	12.0	5	35.5
"	4×10^7	6.6	4	240.0
"	4×10^8	5.4	4	540.0
"	4×10^9	4.8	3	700.0
A/Sn × CBAF$_1$	4×10^6	12.0	5	52.5
"	4×10^7	10.6	5	147.0
"	4×10^8	6.2	4	484.0
CBA	4×10^6	8.2	5	76.0
"	4×10^7	6.2	5	200.0
	4×10^8	4.3	4	594.0
CBA × C57BLF$_1$	4×10^6	7.2	5	208.0
	4×10	7.2	5	329.0
	4×10^8	5.8	4	538.0

* Expressed as maximum number of PFC/10^6 spleen cells at the peak day in a single animal in that dose group
† The A SW *animals used were only 4 weeks of age when tested*
PFC Plaque forming cells

Having established the lag period we now looked at the rate of apperrance of PFC using different doses of antigen, 4×10^6—4×10^9 sheep red cells Each experiment lasted for 6 days after antigen administration and in each dose group 5 animals were sacrificed every day and tested for serum antibodies and PFC in their spleens In *Table I* are summarized the mean doubling times during the exponential phase of increase of PFC in the various strains of mice inoculated with different doses of antigen The results show that the rate of appearance of PFC is directly dependent upon the dose of antigen within the dose range tested If the results obtained with the different strains are pooled for each antigen dose (excluding A SW because of age differences) inoculation of 4×10^6 sheep red cells doubled PFC in 11,7 hours 4×10^7 in 8,7 hours, 4×10^8 in 5,6 hours and 4×10^9 in 5 4 hours This suggests that 4×10^8 sheep erythrocytes are close to the antigen dose giving the maximal rate of antibody production in this system

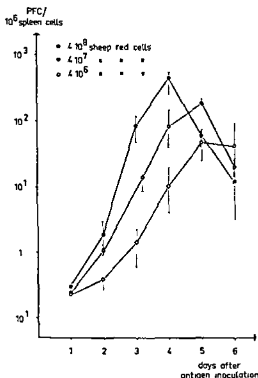


Fig 2

Effect of antigen dose on the rate of increase of PFC This particular experiment was carried out in CBA mice Each dot is representing the geometric means of five animals *The range within each group is indicated*

Table 1 and Fig 2 also show that the time needed to attain maximum number of PFC was also directly related to the antigen dose The higher the antigen dose administered the earlier was the peak values of PFC obtained in the spleens The pooled peak day values were 5,3 days for 4×10^6 sheep red cells, 4,8 days for 4×10^7, 4,3 days for 4×10^8 and 3 5 days for 4×10^9 (only two experiments) The results obtained with 4×10^4 and 4×10^5 sheep red cells followed the same pattern as described above with a still lower rate of increase of PFC and a later peak day The variation with these antigen doses in the experimental groups were, very marked, however, and in order to give any data of a more exact nature the size of the experimental groups must be increased

The relationship between PFC and serum antibodies of various types was now studied The rise of serum antibody titres as measured by haemagglutination and hæmolysis was found to parallell the rise of PFC during the early exponential phase Frequently, there was a somewhat shorter doubling time of antibody titres during the early part of the curve, but in the first day when antibodies were detected was excluded from the calculations there was a complete correspondance between the rise of PFC in the spleen and serum antibody Not only was

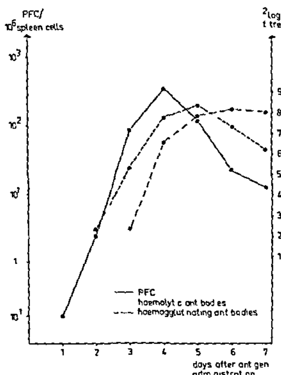


Fig 3

Rate of increase of PFC haemagglutinating and haemolytic antibodies in CBA mice after administration of 4×10^8 sheep erythrocytes at day 0

doubling time somewhat shorter in the early period but there was also a lag period before the serum antibodies appeared as compared to the PFC These results are probably caused by the presence of antigen capable of absorbing serum antibody in the very early phase of antibody synthesis Fig 3 illustrates the relationship between haemagglutinating and haemolytic serum antibodies and PFC during the early exponential phase after injection with 4×10^8 sheep red cells A similar relationship was obtained with the other doses of antigen tested 4×10^6—4×10^9

The ratio between haemolytic and haemagglutinating titres started to change around day 4–5 after the injection of 4×10^8 sheep red cells, the early antiserum being more haemolytic than the later In order to study this shift in reactivity antiserum was passed through Sephadex G-200 gels and Fig 4 illustrates the results obtained with an antiserum obtained 8 days after a primary inoculation of 4×10^8 sheep red cells Two major classes of antibodies were found, the first one appearing in the first protein peak immediately after the void volume whereas the second class appeared in the second protein peak There was never any evidence of any intermediate type of antibodies although they were particularly searched for As can be seen in Fig 4 the first

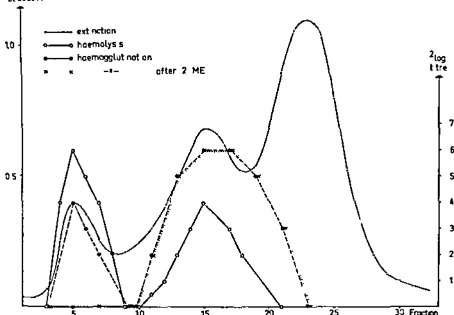


Fig 4

Filtration on Sephadex G 200 and subsequent serological tests on a A SW × CBAF$_1$ anti sheep erythrocyte serum obtained 8 days after a primary challenge with 4 × 10^8 sheep red cells

appearing antibody showed a ratio between haemolytic and haemagglutinating activities that corresponded to the activity of early whole serum after antigen inoculation The haemagglutinating activity of this antibody, which we subsequently will call 19 S antibody, was completely abolished by 2 mercaptoethanol treatment The later appearing serum antibody seemed to correspond in activity with the antibody appearing in the second peak This antibody, which was resistant to 2 mercaptoethanol treatment, will subsequently be called 7 S antibody

Parallel titrations of 19 S and 7 S antibodies and plaque-forming cells of the same animals were then carried out at different time intervals after a primary immunization As shown in Fig 5 there is a close correlation between 19 S titres and number of PFC present whereas the 7 S antibody was not detectable until day 5 6 when the PFC had already started to decline The 7 S titre reached its maximum around day 12–18 after antigen inoculation (4 × 10^8 sheep red cells) and its production persisted for several months, although the values of PFC at that time were close to background values The peak titre of 19 S serum antibody was usually reached one day after the peak of PFC This difference is not due to a cumulation of 19 S antibodies since the 19 S antibodies have a very short half life (6–10 hours) (*Moller & Wigzell*, unpublished)

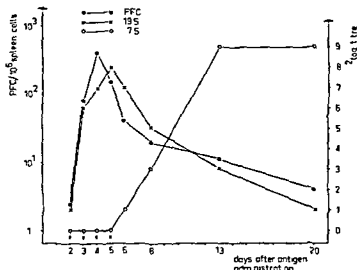


Fig 5

Production of PFC, 19S and 7S antibodies in ASW×CBAF$_1$ mice after a primary challenge with 4 × 10^8 sheep erythrocytes at day 0 Titres expressed in haemagglutinating titres after gel filtration (Sephadex G-200) and subsequent concentrations of the fraction to the original serum volume

It is more likely that extrasplenic 19S synthesis is the cause of this delay of serum antibody titre as a considerable proportion of the PFC can be found outside the spleen on day 4 In our experiments (*Möller & Wigzell*, unpublished) the main extrasplenic sources of PFC have been the peripheral blood and lymph nodes whereas only occasional PFC were ever found in the bone marrow, the thymus or the liver

The results indicate that the PFC detectable in this system produce strongly haemolytic 19S antibodies, whereas 7S production does not result in detectable plaques The failure of 7S producing cells to produce plaques is not due to deficient haemolytic action of 7S antibody in the agar milieu as serial dilutions of 19S and 7S anti-sheep red cell antibodies in Petri dishes prepared as for the plaque technique showed them to be equally efficient in the agar as in the ordinary test tube system It seems more likely that the incompetence of 7S producing *cells is due to quantitative factors at the molecular level of efficiency* Comparisons were made between the titres of 19S and 7S antibodies as measured by the haemolytic and the indirect fluorescent antibody test, respectively The ratio between haemolytic and fluorescent titres was about 100 1 for the 19S antibodies as compared to 1 3 for the 7S antibodies Since the fluorescent antibody test requires the binding of a certain number of antibody molecules for positive staining it follows that the 19S antibody was much more (100 1000?) efficient per molecule *in the haemolytic test system than the 7S antibody*

DISCUSSION

The results presented here are in agreement with the conclusion that the haemolytic plaque technique as used here detects 19S producing cells No evidence of 7S producing cells causing haemolytic plaques was found On the contrary, persisting high 7S haemolysin production occured for several months although the number of PFC was close to background values Parallel titrations of the two classes of antibody in haemagglutinating haemolytic and indirect fluorescent antibody tests and their capacity to inhibit antibody synthesis after passive administration (*Moller & Wigzell* 1965) have provided data suggesting that 19S are many times more efficient (100 1000?) per molecule in causing haemolysis than 7S These results which are in accordance with the results of several others i e *Greenbury Moore & Nunn* 1963 would adequately explain why the 19S but not the 7S producing cells cause haemolytic plaques in the experimental system used

The lag period of antibody formation in this system was found to be of the order of 15 24 hours These results are in agreement with the results of *Jerne Nordin & Henry* 1963 and *Sterzl & Mandel* 1964 who found a lag period in the present system of less than 48 hours Using bacteriphages (*Uhr & Baumann* 1961) or polio virus (*Svehag & Mandel* 1964) still shorter lag periods have been reported the shortest being in the polio virus system where an increased synthesis of antibody was found within four hours after antigen administration The comparatively long lag period in the haemolytic plaque system may be related to the nature of the method requiring the production of a certain minimal amount of antibody per cell before a plaque becomes visible

Jerne Nordin & Henry (1963) using an optimal dose of sheep red cells (4×10^8) did find a close correlation between the early exponential rise of PFC and haemolytic serum titre They also found evidence supporting the idea that at least during the first few days the number of PFC was correlated to the production of antibody sensitive to the treatment of 2 mercaptoethanol We have extended their findings to show that a strong correlation exists in all dose groups at all times between the presence of PFC and 19S serum antibodies Furthermore we have shown that with suboptimal doses of antigen the rise of PFC and 19S serum titres will occur in an exponential way with the rate of increase being dependent upon the dose of antigen administered There was no indication of any divergence between the rise of 19S serum titres and PFC found during the exponential phase which is suggesting that the rate of synthesis of haemolytic 19S per PFC is fairly constant during the exponential phase These results are in agreement with the findings of *Strander* (1964 unpublished) that the mean diameter of the haemolytic plaques is the same during the first three to four days whereafter they tend to decrease in size Thus there was no evidence of an exponential increase in the absolute rate of antibody

synthesis per cell as suggested by *Uhr & Finkelstein* (1963) However, recent reports in another system (*Nossal et al* 1964) indicate that in a rat-anti Salmonella system in an increase in the rate of 19 S antibody synthesis per cell with time, the maximum being reached on day 6 after a primary immunization

The minimum doubling time of PFC and 19 S serum titres was found to be 5 4 hours when an optimal dose of sheep red cells was administered which is close to the values obtained by *Jerne & Nordin* (1963) This short doubling of serum antibody titres has also been found in other systems (*Svehag & Mandel* 1964, *Uhr & Finkelstein* 1963) Reported doubling times for lymphoid cells after immunization using autoradiography have been as short as 7 hours (*Capalbo & Makinodan* 1962) whereas certain ascites lymphoma cells divide every 5,2 hours during their exponential growth (*Revesz & Klein* 1954) These figures closely parallell the maximal rate of increase of PFC in our system Furthermore, frequency distribution studies on the concentration of PFC inside spleen colonies indicate a clonal growth of the PFC inside single colonies (*Celada & Wigzell* 1966) It is therefore considered *likely that the exponential rise of PFC is mainly due to cellular proliferation*, the rate of which is *directly dependent upon antigen concentration*

Other possibilities, however, for explaining parts of the exponential rise cannot be ruled completely Thus, *it is possible that some PFC start to synthesize antibodies in the absence of proliferation after having received information for antibody synthesis* This possibility is suggested by the findings of *Fishman* (1961) and by recent claims that RNA from spleens of mice immunized against sheep red cells can confer the ability to produce haemolytic plaques on non-immune spleen cells (*Cohen & Parks* 1964, *Friedman* 1964) According to this alternative, the informative agent(s) *must be produced at an exponential rate* however, unless a multi-component system is postulated Another possibility is that low concentrations of antibody directed against the antigen may increase the rate of recruitment of plaque-forming cells by increasing the immunogenicity of the antigen There are some indications in the haemolytic plaque system that low-titered 19 S antibodies administered together with the sheep erythrocytes might occasionally enhance the rate of increase of PFC (*Moller & Wigzell* 1965)

SUMMARY

The kinetics of the primary immune response has been followed in mice immunized against sheep red cells Single, antibody-producing cells have been studied using a haemolytic plaque assay whereas serum antibodies have been determined using haemolysis, haemagglutination and indirect fluorescent antibody tests The lag period for plaque forming cells (PFC) to increase after antigen administration was about

20 hours Thereafter, the number of PFC increased at an exponential rate, the doubling time being dependent upon the dose of antigen give The doubling time varied between 11,7 hours after 4×10^6 sheep red cells and 5 4 hours after giving 4×10^9 In addition, higher doses of antigen caused the peak of PFC to appear earlier and did also increase the absolute number of PFC The rise and fall of PFC was paralleled by the production of 19S antibodies whereas the production of 7S antibodies, if anything, had a depressive effect on the number of PFC Different parallel titrations of 19S and 7S antibodies in the different serological systems indicate that the 19S antibody in this system is 100–1000 times more efficient per molecule in haemolysis as compared to 7S

It is considered that the immune response against increasing doses of antigen in the investigated system is taking place through an increase in the number of antibody producing cells and not through an increase in the rate of antibody synthesis per cell The rate of increase of plaque-forming cells was found to be directly dependent upon antigen concentration

REFERENCES

Bradley S C & Watson D W Production of neutralising antibody by mice inoculated with actinophage J Immunol *90* 782 787 1963

Capalbo F E & Makinodan T Doubling time of mouse spleen cells during the latent and log phases of primary response J Immunol *92* 234 242 1962

Celada F & Wigzell H Immune responses in spleen colonies I Clonal distribution of haemolytic plaque forming cells Immunology *10* 231 243 1966

Cohen E P & Parks J J Antibody production by nonimmune spleen cells incubated with RNA from immunized mice Science *144* 1012 1013 1964

Eagle H Propagation in a fluid medium of a human epidermoid carcinoma strain Proc Soc Exp Biol Med *89* 362 364 1955

Fishman M Antibody formation *in vitro* J Exp Med *114* 837–856 1961

Friedman H Antibody plaque formation by normal mouse spleen cell cultures exposed *in vitro* to RNA from immune mice Science *146* 934–935 1964

Greenbury C L Moore D H & Nunn L A C Reaction of 7S and 19S components

and AB red cells Immunology

by single antibody producing

ique technique for recognizing

Wistar Institute Press Phila delphia 109 125 1963

Moller G & Wigzell H Antibody synthesis at the cellular level Antibody induced suppression of 19S and 7S antibody response J Exp Med *121* 969 989 1965

Moller G Demonstration of mouse isoantigens at the cellular level by the fluorescent antibody technique J Exp Med *114* 415 434 1964

Nossal G J V Szenberg A Ada G L & Austin C A Single cell studies on 19S antibody production J Exp Med *119* 485–502 1964

Revész L & Klein G Quantitative studies on the multiplication of neoplastic cells *in vivo* II Growth curves of three ascites lymphomas J Nat Cancer Inst *14* 253 272 1954

Ster I J & Mandel I Estimation of the inductive phase of antibody formation by plaque technique Fol Microbiol *9* 173 176 1964

Stimpfling J H The use of PVP as a developing agent in mouse haemagglutinati tests Transplant Bull 27 109 111 1962

ıehag S E & Mandel B The formation and properties of polio virus neutralizing antibodies I 19S and 7S antibody formation Differences in kinetics and antigen dose requirements for induction J Exp Med *119* 1 19 1964
hr J W & Baumann J B Antibody formation I The suppression of antibody formation by passively administered antibody J Exp Med *113* 935 957 1961
hr J W & Finkelstein M S Antibody formation IV Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacterophage O X 174 J Exp Med *117* 457–477 1963
rso P & Makinodan T The roles of cellular division and maturation in the formation of precipitating antibody J Immunol *90* 897 907 1963

Acta path et microbiol scandinav 66 541 550 1966

The Department of Antibiotics Statens Seruminstitut Copenhagen Denmark

THE *IN VITRO* ACTIVITY OF CEPHALORIDINE

Spectrum Routine Sensitivity Tests, Cross Resistance and Inactivation

By

PER HYE KNUDSEN

Received 23 ix 65

Cephaloridine[1] is a semisynthetic antibiotic belonging to the cephalosporins It is made chemically from cephalosporin C, a natural antibiotic produced by the mould *Cephalosporium acremonium* The structure resembles that of penicillin (1, 9 14)

Cephaloridine is a white powder, readily soluble in water, the solution being slightly acid (pH 4 5 to 5) Aqueous solutions are stable at this pH for at least 4 weeks at 4° C in the dark and more stable than solutions made more alkaline The powder is stable in the refrigerator at 4° C

Cephaloridine should be given by parenteral injection as the absorption after oral administration is poor (9) There appears to be no significant degree of protein binding in serum (4) It is rapidly excreted in the urine as unchanged drug (9 14)

In the present paper the purpose has been to determine the activity of cephaloridine against various common pathogenic bacteria to establish a method for routine sensitivity testing to investigate the degree of drug inactivation caused by penicillinase producing strains of *Staphylococcus aureus*, and to evaluate any cross resistance between cephaloridine and other penicillins

MATERIAL AND METHODS

Using the plate dilution method the 50 per cent inhibitory concentration (IC 50) of cephaloridine has been determined against 150 strains belonging to 14 genera These strains consist of a few type cultures and of many strains from routine examination of faeces urine sputum and pus from patients selected so as to be representative of most of the common pathogenic bacteria

150 consecutive strains of *Staph aureus* freshly isolated from clinical material were used in the evaluation of cross resistance between cephaloridine and other penicillins

---

[1] Cephaloridine was placed at my disposal by courtesy of Ercopharm Ltd Copenhagen

Plate Dilution Method

5 per cent blood agar containing cephaloridine in two-fold dilution steps from 100 µg/ml to 0.025 µg/ml, and a control without antibiotic was poured into 9 cm Petri dishes. The plates were stored at 4° C and used within 4 days from day of preparation. The plate was divided into 16 sections and each section was seeded with one loopful of a 20-hour serum broth culture, diluted with 0.9 per cent NaCl as follows:

Gram-negative rods 1:1000
Gram-positive cocci 1: 100
Gram-positive rods 1: 100

The plates were incubated for 18–20 hours at 37° C; subsequently growth on the plates was read and compared with growth on the control plate, the growth being indicated by 4, 3, 2, 1 and 0, corresponding to 100, 75, 50, 25 and 0 per cent growth as compared with growth on the control plate (12). IC 50 was calculated by the method of *Karber* (7).

Agar Prediffusion Method

The method used was "Method 5" (15), using 6 mm paper discs containing 50, 25 and 5 µg of cephaloridine on 10 per cent blood agar plates without peptone but with the addition of 1 per cent glucose. After a prediffusion period of 20 hours at room temperature the discs were removed and the plate inoculated with 0.1 ml of the above culture dilution using a bent glass rod for spreading. The inhibition zones were measured after incubating for 20 hours at 37° C.

Inactivation of Cephaloridine

The test strains were incubated at 37° C overnight on 5 per cent horse blood agar plates containing 0.01 µg/ml cephaloridine for enzyme induction. Cultures were harvested in 4 ml of broth so as to make a milky suspension and to this 4 ml of a 200 µg/ml cephaloridine solution were added making the concentration 100 µg/ml. After incubation for varying periods samples were taken out, and the enzyme effect interrupted by heating to 100° C on a water bath for 1 minute. The remaining drug activity was determined using the agar cup method described by *Vesterdal* (16). *Staph. aureus* FDA 209 P was used as test organism. An uninoculated tube containing 100 µg/ml of cephaloridine in broth was included in the experiment as a control.

RESULTS

Activity of Cephaloridine

The activity of cephaloridine against various gram-positive bacteria is shown in Table 1. Pneumococci, haemolytic streptococci group A and a penicillin-sensitive *Staph. aureus* were inhibited by a concentration of 0.025 µg/ml or less. *Staph. albus* strains also showed high sensitivity. *Streptococcus faecalis* was the most resistant of the gram-positive bacteria. The activity against *Staph. aureus* showed a dependency of the penicillin and oxacillin sensitivity of the strain: the more resistant the strain was to these two penicillins, the higher concentration of cephaloridine was required for inhibition.

The results obtained with a number of strains of gram-negative rods are recorded in Table 2. Strains of *Salmonella*, *Shigella* and *Pasteurella pseudotuberculosis* were sensitive to cephaloridine. Six of the 8 *E. coli* strains were sensitive, the remainder being resistant to > 100 µg/ml. *Proteus mirabilis* was borderline sensitive, while the other *Proteus* species were resistant. *Klebsiella* was inhibited in concentrations varying from 5 to > 100 µg/ml.

TABLE 1

The Activity of Cephaloridine against 75 Strains of Gram Positive Bacteria

Species	50 per cent inhibitory concentration in μg/ml				
	≤0.05	0.05–0.2	0.2–0.8	0.8–3.2	3.2–12.5
Staph. aureus penicillin sensit	1				
Staph aureus penicillin resist	2	8			
Staph aureus oxacillin resist			7	14	
Staph albus	4	3			
Pneumococcus	8				
Strep haem Gr A	5				
Strep viridans	3		3		1
Strep faecalis				1	7
Listeria monocytogenes			4		
Corynebact dipht	3				
Bact subtilis				1	

The table shows the number of strains inhibited by the various concentrations. Strains on border values are recorded in the sensitive group

TABLE 2

The Activity of Cephaloridine against 75 Strains of Gram Negative Rods

Species	50 per cent inhibitory concentration in μg/ml				
	≦0.8	0.8–3.2	3.2–12.5	12.5–50	>50
Escherichia coli		6			[illegible]
Klebsiella			5	2	1
Proteus mirabilis		2	5		
Proteus morgani					4
Proteus rettgeri				2	2
Proteus vulgaris					8
Providencia					4
Pseudomonas aeruginosa					
Salmonella	1	7			
Shigella		8			
Past pseudotuberculosis	4				
Bact. anitratum				2	2

(For further explanation see Table 1)

Routine Determination of Bacterial Sensitivity to Cephaloridine Using the Prediffusion Method

The classification of bacteria into different sensitivity groups is based on the concentrations of the antibiotic which can be achieved in serum. Stewart & Holt (14) found cephaloridine concentrations of 2 to 30 μg/ml persisting for 3–4 hours after intramuscular administration of 250–500 mg four times daily. In other studies (9, 10) in which serum levels have been determined 1 hour after a single injection of 500 mg the concentrations averaged 10–20 μg/ml. Strains with an IC 50 below one third to one fifth of the serum concentration after normal doses are considered sensitive, whereas strains which are not inhibited by concentrations attainable in clinical routine are considered resistant.

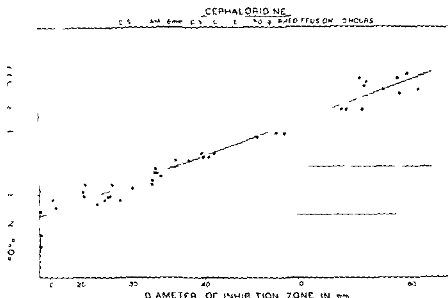


Fig 1

The correlation between values obtained by the plate dilution method and the prediffusion method Each point indicates one strain tested twice The horizontal lines show the limits between sensitivity groups

In this study a strain with an IC 50 below 3 0 μg/ml was considered sensitive, while strains with an IC 50 of more than 25 μg ml were considered resistant

The correlation between results obtained by the plate dilution method and the prediffusion method has been recorded in Fig 1, and the test was carried out as follows

46 strains were selected from the 150 strains used in the determination of IC 50 The strains were selected in such a way that their IC 50 were distributed evenly from ≦ 0 025 to > 100 μg/ml The inhibition zones for these strains were determined by the prediffusion method using discs containing 50, 25 and 5 μg The corresponding values were plotted in a diagram for each disc content Fig 1 shows the results for 50 μg per disc This amount per disc was chosen for routine sensitivity testing as it gives no inhibition zones with strains of IC 50 ≧ 25 μg ml and zones of more than 35 mm with sensitive strains When using the squared values of the inhibition zones the points were seen to be distributed along a straight line which was then fitted to the points by the eye This line will give a sufficiently reliable correspondence between the inhibitory concentration and the diameter of the inhibition zone

Effect of Inoculum Size

The effect of inoculum size on the activity of cephaloridine against *Staph aureus*, *Proteus mirabilis* and *Klebsiella* has been tested and

TABLE 3

Effect of Inoculum Si e on Sensitivity of Staph aureus *to Cephaloridine*

Staph aureus	Inoculum	50 per cent inh bitory cc ncentration in µg/ml 0 025 0 05	0 05 0 1	0 1 0 2	0 2 0 4	0 4 0 8	0 8–1 6	1 6–3 2	3 2 6 25	6 25–12 5
Penicillin sensitive	1/100	1								
	undil	1								
Penicillin resistant	1/100	2	7							
	undil		1	1	3	3	1			
Oxacillin resistant	1/100					7	14			
	undil							6	13	2

(For further explanation cf Table 1)

TABLE 4

Effect of Inoculum Size on Sensitivity of Proteus mirabilis *and* Klebsiella

Species	Inoculum	50 per cent inhibitory concentration in µg/ml 1 6 3 2	3 2 6 25	6 25 12 5	12 5 25	25 50	50–100	> 100
Proteus mirabilis	1/100	2	5					
	undil			7				
Klebsiella	1/100		1	4	2			
	undil						4	3

(For further explanation cf Table 1)

the results are recorded in Tables 3 and 4 No effect was seen in the penicillin-sensitive strain of *Staph aureus*, while the effect on the penicillin resistant/oxacillin-sensitive strains was an increase in inhibitory concentrations of 2 to 10 fold and a similar effect was found in the oxacillin resistant strains

The sensitivity of the *Klebsiella* strains decreased 5 to 20 times The effect on *Proteus mirabilis* was only minor with a 2 to 3 fold decrease, corresponding to their sensitivity to high doses of penicillin and ampicillin

Inactivation of Cephaloridine by Staphylococcus aureus

24 strains were tested for their ability to inactivate cephaloridine The strains were equally distributed into 3 groups

1 Penicillin-sensitive
2 Penicillin-resistant/oxacillin-sensitive
3 Penicillin resistant/oxacillin-resistant

The investigations were performed as described under methods, samples were taken out after 1, 4, and 24 hours' incubation and the remaining drug activity was determined The penicillin-sensitive strains were unable to inactivate cephaloridine, whereas strains being penicillin resistant inactivated cephaloridine completely in 4 hours, some of them even in 1 hour, their sensitivity to oxacillin being without influence on the speed of inactivation

Inactivation of Oxacillin by Staphylococcus aureus

29 strains were tested for inactivation of oxacillin in the same way as described above Eight penicillin-sensitive strains were all unable to inactivate oxacillin Of 20 penicillin resistant/oxacillin-sensitive strains all were able to decrease the activity, but the inactivation proceeded more slowly than inactivation of cephaloridine The remaining drug activity after 4 hours rated from practically unchanged to 20 per cent, the average being 50 per cent, and only 8 strains were able to inactivate oxacillin completely in 24 hours One oxacillin resistant strain acted in the same way as the previous group leaving no activity of oxacillin after 24 hours

Correlation of Activity of Cephaloridine and of four other Penicillins against Staphylococcus aureus

150 consecutive strains of *Staph aureus* were tested using the 20 hour prediffusion method against cephaloridine, benzylpenicillin, ampicillin oxacillin and cloxacillin The correlation between benzylpenicillin and ampicillin was complete and so was the correlation between oxacillin and cloxacillin Accordingly only the correlation between benzylpenicillin cephaloridine and oxacillin may be commented upon

(Table 5) The following distribution was found among the 150 strains 32 were sensitive to benzylpenicillin, 135 sensitive to cephaloridine and 146 sensitive to oxacillin The sensitivity to cephaloridine was found to be only moderate in 13 strains, and 2 strains were resistant, not only to cephaloridine but also to oxacillin 11 of the moderately sensitive strains were sensitive to oxacillin, the remainder being resistant

TABLE 5

150 Consecutive Strains of Staph aureus *Grouped According to Their Sensitivity to Cephaloridine Benzylpenicillin and Oxacillin*

Sensitivity pattern			Number of strains
C 3	P 3	Ox 3	32
C 3	P 0	Ox 3	103
C +	P 0	Ox 3	11
C +	P 0	Ox 0	2
C 0	P 0	Ox 0	2
			150

C = Cephaloridine 3 = Sensitive
P = Benzylpenicillin + = Moderately sensitive
Ox = Oxacillin 0 = Resistant

TABLE 6

22 Strains of Gram Negative Rods Grouped According to Their Sensitivity to Benzylpenicillin Ampicillin and Cephaloridine

Sensitivity pattern			Number of strains	Species
P +	A 3	C +	2	*Proteus mirabilis*
P +	A +	C +	4	
P 0	A +	C 3	3	*E coli*
P 0	A +	C +	2	
P 0	A 0	C 3	1	
P 0	A 0	C 0	2	
P 0	A 0	C 0	8	*Klebsiella*

P = Benzylpenicillin 3 = Sensitive
A = Ampicillin + = Moderately sensitive
C = Cephaloridine 0 = Resistant

Correlation of Activity of Cephaloridine, Benzylpenicillin and Ampicillin against Gram-Negative Rods

The sensitivity of 8 *E coli*, 8 *Klebsiella* and 6 *Proteus mirabilis* strains were determined simultaneously against cephaloridine, benzyl penicillin and ampicillin with the 20 hour prediffusion method The *Klebsiella* strains were all resistant to the three antibiotics Penicillin resistance was found with all *E coli* strains and 3 of 8 strains were also resistant to ampicillin, the remainder being only moderately sensitive

Against cephaloridine 4 strains showed full sensitivity, 2 were moderately sensitive and 2 resistant The *Proteus mirabilis* group was moderately sensitive to all three antibiotics but for 2 strains which showed full sensitivity to ampicillin (Table 6)

DISCUSSION

The spectrum shown in Tables 1 and 2 is in good agreement with previous reports (4, 9, 10, 14) Cephaloridine is seen to be a broad-spectred antibiotic, which can be used in infections caused by most gram-positive bacteria and by some gram-negative bacteria too, i e *Salmonella*, *Shigella* and a number of strains belonging to *E coli*, *Klebsiella* and *Proteus mirabilis*

The inoculum effect on the activity of cephaloridine, which has been shown in this study with strains of *Klebsiella* and penicillin-resistant *Staph aureus*, is of the same magnitude as previous described (4) The inoculum effect has been regarded as being due to inactivation by penicillinase (3, 5)

It is now generally agreed that the so-called penicillinase-resistant penicillins are inactivated by staphylococcal penicillinase (2, 5, 6, 8) *Ayliffe & Barber* (2) found that the inactivation of methicillin depended upon the degree of penicillinase production by the organism, so that an active penicillinase producer, whether methicillin-resistant or not inactivated a considerable portion of methicillin, whereas the weak penicillinase producer caused no inactivation The present work showed that strains of *Staph aureus* which were unable to inactivate cephaloridine belonged to the non-penicillinase producing strains, and these strains were also unable to inactivate oxacillin, whereas penicillinase producing strains were able to inactivate both antibiotics

A comparative study of the inactivation rate of the so-called penicillinase-resistant penicillins by staphylococcal penicillinase has been made by different groups *Nayler et al* (11) compared methicillin, cloxacillin and oxacillin and found methicillin to be the most stable, cloxacillin to be slightly less stable than methicillin, but a little more stable than oxacillin *Smith et al* (13) had similar results *Eriksen & Erichsen* (6) included a cephalosporin, cephalothin, in their study and they found that these penicillins were all inactivated, but the inactivation proceeded at different rates The isoxazolylpenicillins were inactivated at a particularly high rate, methicillin much more slowly and cephalothin was something in between In the present investigation a comparison was made between cephaloridine and oxacillin, and cephaloridine was found to be inactivated more easily than oxacillin By correlating these results a rating of the inactivation of the "penicillinase-resistant" penicillins by staphylococcal penicillinase could be made Methicillin is the most stable then comes cephalothin Of the isoxazolylpenicillins cloxacillin seems to be more resistant to peni-

cillinase than oxacillin but both are less stable than methicillin and cephalothin The last in the rating is cephaloridine

No cross resistance was found between cephaloridine and any of the other penicillins tested Against *Staph aureus* it was found to be less active than oxacillin (15 strains with decreased sensitivity to cephaloridine compared with only 4 oxacillin resistant) Against the gram negative rods cephaloridine had an advantage above ampicillin against *E coli* but against *Proteus mirabilis* and *Klebsiella* it did not offer any advantage

SUMMARY

The *in vitro* activity of cephaloridine has been determined by the plate dilution method against 150 strains belonging to 14 genera and the findings have been correlated with the results from a 20 hour pre diffusion assay in order to work out a method for routine sensitivity testing

Cephaloridine is a broad spectred antibiotic which inhibits most gram positive bacteria within limits of concentrations achievable in serum with therapeutic doses It can also be used in infections caused by many of the gram negative rods i e *Salmonella* *Shigella* and a number of strains belonging to *E coli* *Klebsiella* and *Proteus mirabilis*

A rating of the stability of the penicillinase resistant penicillins to staphylococcal penicillinase is made showing the following order with the most stable one named first methicillin cephalothin cloxacillin oxacillin and cephaloridine

More strains of *Staph aureus* are found with decreased sensitivity to cephaloridine than to oxacillin Against *E coli* it is found to have an advantage above ampicillin

No cross resistance has been demonstrated between cephaloridine and other penicillins

REFERENCES

1 *Abraham E P & Newton G G F* New penicillins cephalosporin C and penicillinase Endeavour 20 92 100 1961
2 *Ayliffe G A J & Barber M* Inactivation of benzylpenicillin and methicillin by hospital staphylococci Brit med J II 20 205 1963
3 *Barber M & Waterworth P M* Antibacterial activity of the penicillins Brit med J I 1159 64 1962
4 *Barber M & Waterworth P M* Penicillinase resistant penicillins and cephalosporins Brit med J II 344 349 1964
5 *Eriksen K R & Erichsen I* Resistance to methicillin isoxazolylpenicillins and cephalothin in *Staph aureus* Acta path et microbiol scand nav 62 255–75 1964
6 *Eriksen K R & Erichsen I* Inactivation of methicillin oxacillin cloxacillin and cephalothin by staphylococcal penicillinase Acta path et microbiol scand nav 62 399–408 1964
7 *Finney D J* Statistical methods in biological assay London 1952 p 524
8 *Knox R & Smith J T* Stability of methicillin and cloxacillin to staphylococcal penicillinase Brit med J II 205–207 1963
9 *Muggleton P W O'Callaghan C H & Stevens W K* Laboratory evaluation of a new antibiotic—cephaloridine (ceporin) Brit med J II 1234 3 1964

10 *Murdoch J McM Speirs C F Geddes A M & Wallace E T* Clinical trial of cephaloridine (ceporin) a new broad spectrum antibiotic derived from cephalosporin C Brit med J *II* 1238 40 1964
11 *Nayler J H C Long A A W Brown D M Acred P Rolinson G N Batchelor F R Stevens S & Sutherland R* Chemistry toxicology, pharmacology and microbiology of a new acid stable penicillin resistant to penicillinase (BRL 1621) Nature *195* 1264–67 1962
12 *Reyn A Bentzon M W & Ericsson H* Comparative investigations of the sensitivity of N *gonorrhoeae* to penicillin Acta path et microbiol scandinav 57 235 255 1963
13 *Smith J T Hamilton Miller J M T & Knox R* Isoxazolyl penicillins and penicillinase Nature *195* 1300–01 1962
14 *Stewart G T & Holt R J* Laboratory and clinical results with cephaloridine Lancet *II* 1305–09 1964
15 *Thomsen V F* Correlation of the plate dilution method to the agar diffusion method (disc and tablet methods) with a special view to the importance of prediffusion Acta path et microbiol scandinav 54 107 120 1962
16 *Vesterdal J* The agar cup method for the estimation of penicillin Acta pharmacol (Kbh) 2 9 21 1946

Acta path et microbiol scandinav 66 551 559 1966

The Municipal Virological Laboratory Gothenburg Sweden

THE VIRUS INACTIVATING CAPACITY OF SEA WATER

By

SIGYN MAGNUSSON, CARL ERIK HEDSTROM and ERIK LYCKE

Received 16 x 65

It has been known for a long time that pathogenic enteric bacteria have a short survival time in fresh sea water Some reports suggest that this is due to a bactericidal action of the sea water The many studies on the limited survival of terrestrial microorganisms in sea water and the proposed reasons for it have been reviewed by *Greenberg* (1)

The present report demonstrates that the infectivity of a number of viruses is unstable in sea water The possible influence on virus infectivity of some inorganic compounds of sea water and of light is investigated and discussed In addition the kinetics of the inactivation is studied in some detail

MATERIAL AND METHODS

Viruses Unless otherwise stated one batch of poliovirus type 3 Saukett was used The virus suspension employed was produced in monkey kidney cell cultures and cleared by low speed centrifugation The infectivity titre of the suspension was 6 5 TCD_{50} per 0 1 ml

In some experiments a number of other viruses were used namely poliovirus type 1 SBL 1423 53 and type 2 MEF 1 Coxsackie virus type B_3 ET 652 H adenovirus type 7 GOTA 173/64 ECHO virus type 32 PR 10 vaccinia virus SBL BT 604/63 influenza virus type A_2 GOT 485/64 *E coli* phage T 1 Suspensions of the polio ECHO and Coxsackie viruses were produced in monkey kidney cell cultures the adenovirus suspension in HeLa cells and the influenza and vaccinia virus suspensions in embryonated chicken eggs

The phage suspensions was obtained from infected broth cultures of *E coli* B cells according to the technique employed by *Kallings* (2) The phage suspension using sodium dextrane sulphate and methyl tsson (3)

kidney cells and HeLa cells were cultured in on to which were added 0 5 per cent lactalbumin hydrolysate 7 per cent calf serum 100 I U of penicillin and 100 micrograms of streptomycin per ml

Assay of virus infectivity Cultures of trypsinized monkey kidney cells were used for titration of infectivity of animal viruses with the exception of the aden virus infectivity which was titrated in HeLa cells Tube titrations using serial 10 fold dilutions with 5 tubes per dilution and 0 1 ml of inocula were used Titres were recorded as TCD_{50} per 0 1 ml and were calculated according to *Karber* (4)

This study was supported by a grant from the *Swedish Association against poliomyelitis*

The infectivity of the T1 phage was tested on *E coli* B bacteria by plaque titrations according to *Kallings* (2)

Sea water samples If not otherwise stated the batches of sea water originated from localities in the Gothenburg archipelago They were stored at +4° C in glass or plastic containers until use

Experimental procedure Untreated sea water at 25° C was added to the virus suspensions in the proportions 100 1 The infected sea water was then incubated at 23° C. Samples were drawn after one hour and daily for 8 days and tested for residual virus infectivity As heating of the sea water to temperatures above 45° C for one hour was found to destroy its virus inactivating capacity (5), heat treated water (90° C for one hour) was used for obtaining adequate controls Heat treated water was used as a control in parallel with the unheated water samples in the tests The virus inactivating capacity (VIC) of a sea water specimen investigated was defined as the difference in the infectivity titres of samples taken 1 hour and 8 days after the start of the experiment

Na Cl and osmolarity Concentrations of Na^+ and Cl were determined flame photometrically Osmolarity was estimated by freezing point determinations

RESULTS

Demonstration of a virus inactivating capacity (VIC) in sea water Under the experimental conditions applied the loss of infectivity encountered when virus was suspended in heat-treated sea water (controls) never amounted to 2 log units and as a rule was less than one log unit It was therefore considered that a significant VIC of a tested untreated sea water specimen was demonstrated if the reduction in infectivity exceeded 2 log units

TABLE 1
The Virus Inactivating capacity (VIC) of 26 Surface Water Samples from Different Localities (Fig 1)

Sample	VIC	Sample	VIC
1	1 7	14	2 0
2	3 2	15	4 6
3	1 1	16	4 4
4	1 6	17	2 7
5	1 6	18	3 6
6	2 9	19	3 7
7	3 4	20	3 8
8	3 7	21	3 0
9	1 6	22	2 6
10	3 6	23	2 6
11	3 6	24	4 3
12	0 8	25	3 9
13	3 8	26	0 5

Nos 1 10 samples from the Baltic Sea Nos 11 13 25 samples from the North Sea No 12 river water No 26 lake water

Table 1 shows that out of the 26 tested specimens obtained from different localities 5 possessed no significant VIC All of these specimens originated from various localities in the Baltic (Fig 1) Each of the 14 specimens from the west coast of Sweden and the North Sea showed a significant VIC Waters sampled from different depths of

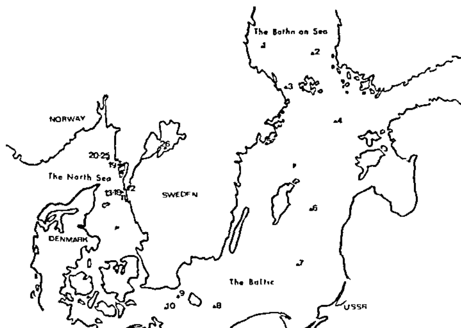


Fig 1
Localities for sampling of sea water (cf Table 1)

the North Sea had significant VIC values (Table 2) Specimens from rivers and lakes tested, on the contrary, showed no VIC These observations suggested, *i a* a possible correlation between the occurrence of VIC and the NaCl concentration of the water

TABLE 2
The Virus Inactivating Capacity (VIC) of Sea Water Sampled from Different Depths of the North Sea

Depth in meters	VIC
Surface	3 8
10	3 7
50	3 0
100	3 0
190	2 7

As indicated in Table 3, there was also a certain correlation discernible between the NaCl concentration and the osmolarity, on the one hand, and the VIC, on the other hand, of 6 waters investigated However, there was no direct proportionality between VIC and the concentration of NaCl or the osmolarity The same VIC was demonstrable in specimens with a 3-fold difference in the NaCl concentration whereas other specimens with almost the same salinity differed in VIC with 2 log units

Effect on VIC by varying some inorganic compounds Results obtained by experimental variation of the NaCl concentration are ac-

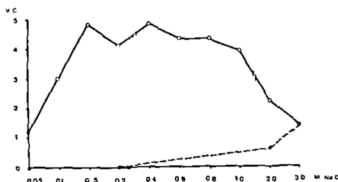


Fig 2

Effect of NaCl concentration on the virus inactivating capacity (VIC) of sea water

counted for in Fig 2 The salinity of the untreated water specimen used corresponded to 0 2 M NaCl Concentrations above this value were obtained by addition of NaCl whereas lower concentrations were achieved by "diluting" the sea water with distilled water Within a range of 0 1–2 0 M NaCl the VIC exceeded 2 log units Controls using heat-treated water or a buffered 0 2 M NaCl solution as diluent had no demonstrable effect on the VIC The reduction in VIC observed with sea water below a 0 1 M concentration of NaCl seemed thus correlated to the decrease in salinity At the concentrations of NaCl tested and at pH 7 the infectivity of the poliovirus strain used was relatively stable using heat treated sea water or salt solutions made in the laboratory and only at a concentration of 3 M NaCl a significant reduction in infectivity was encountered

TABLE 3

The Virus Inactivating Capacity (VIC) the Concentrations of Na Cl and the Osmolarity of 6 Sea Water Samples Investigated

VIC	Na^+	Cl^+	mOsm/l
3 7	92	118	219
3 6	303	337	644
3 6	110	142	230
2 9	89	112	209
1 6	82	107	208
1 6	82	104	196

The concentrations of Na^+ and Cl are expressed as milli equivalents *per litre of water*

The addition of 0 06 M KI or 0 08 M Na_2SO_3 to heat-treated water did not supply the heat-treated water with virus inactivating capacity Determinations of the oxidation potentials of various sea water specimens according to the technique used by *Lund & Lycke* (6) resulted in values below 200 mV in all tests At these levels there seems to be no noticeable oxidative inactivation of polio virus infectivity

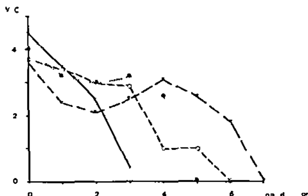


Fig 3
Effect of dilution on the virus inactivating capacity (VIC) of sea water

Effect on the VIC by exposure of water to light The VIC of a batch of sea water kept in darkness or exposed to light during the incubation period was studied Special caution was taken to avoid light exposure of dark samples before titration of infectivity The VIC demonstrated was of the same order whether or not the water was exposed to light

Effect of dilution on the VIC Three batches of sea water were diluted in serial 10 fold steps using heat treated water as diluent Virus was added to each dilution and the VIC was tested As the end point the dilution which still afforded a reduction in infectivity of 2 log units or more was chosen The logarithm of this dilution is referred to as the dilution capacity of the water

TABLE 4
The Relation of the Virus Inactivating Capacity (VIC) to the Dilution Capacity (DC) of 4 Sea Water Samples

DC	VIC
2	4 5
3	3 7
4	4 0
5	3 6

Table 4 shows that the dilution capacity of the waters could vary considerably although the VIC of the undiluted water differed less than one log unit It might be assumed (Fig 3) that a certain concentration of the factor(s) responsible for the virus inactivation was required before a significant VIC could be recorded The VIC value seemed therefore scarcely useful for estimation of the concentration of the responsible factors once these exceeded the concentration required for obtaining a significant VIC

The course of virus inactivation in waters with different dilution capacities The course of the virus inactivation in waters with different dilution capacities is demonstrated in Fig 4 The inactivation curves

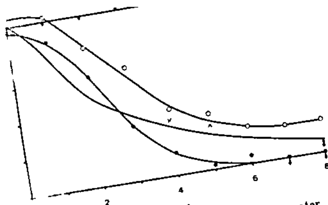


Fig 4
...ic virus inactivation in sea water ... ed against the incubation time at 25° in days ... s represent results obtained with three different ... capacities of 2 3 and 5 respectively (see text) ... ults of control (heat treated sea water)

...tial lag phases and a gradually reduced ... atter part of the observation period As ... limited to explain these phenomena ... irse as well the rate of inactivation ... ent upon the dilution capacity i e ... tivating factor(s) in the sea water

...ABLE 5
... Capacity (VIC) of a Sea Water Sample ... re Different Viruses

	VIC
	2 0
	3 2
	4 5
3	3 2
... a kie B	4 8
ECHO 3?	2 6
Aden 7	2 5
... a	2 9
... h ... T1	0 3

Stability of some different viruses in sea water Two animal DNA and 6 RNA viruses were studied using a water with a dilution capacity of 2 All of these viruses were found unstable in the water used In contrast to this observation a bacterial virus (T1 phage) seemed resistant (Table 5) This was confirmed by testing polio type 3 and the T1 phage simultaneously However when the stability of the T 1 phage in waters with different dilution capacities was studied it was found that if a water with a high capacity was used a marked reduction in phage infectivity could be obtained This would indicate that only a

relative difference in sensitivity existed between the phage and the animal viruses

DISCUSSION

In a previous study (7) it was incidentally observed that the infectivity of polio virus suspended in untreated sea water was reduced at a markedly high rate. The virus strain used seemed to be considerably more unstable in sea water than in salt solutions with the same salinity and pH as sea water prepared in the laboratory.

A comparison was made between the incubation time required for the appearence of cytopathic changes in cell cultures inoculated with virus in samples of sea water and the incubation time necessary when virus was added to the cultures with sea water samples highly diluted with a buffer solution. Provided cultures inoculated with the same doses of infective virus were compared, no difference in incubation times were observed. Thus the decrease in titres of residual virus infectivity after exposure of virus to sea water was apparently not due to an effect of sea water on the sensitivity of the cell cultures used. Viral infectivity seemed therefore to have been destroyed or masked. The effect was referred to as inactivation of virus.

Although a bactericidal effect of sea water has been observed and discussed for a long time the stability of viruses in sea water seems to have attracted little interest. *Plissier* and coworkers (8, 9, 10) have compared the inactivation rates of viruses suspended in sea water and in fresh water. They reported that the inactivation rate of polio virus in sea water or in tapwater of the city of Nice was essentially of the same order. At 17° C the inactivation in tap water and in sea water corresponded to "half life" values ranging from 14.71 and 17.51 days respectively (10). *Plissier et al.* seem, however, to have exclusively used sea water which was sterilized by Seitz filtration or by chlorination before the addition of virus. According to our observations both these means eliminate the particular virus inactivating capacity of the sea water.

The concentrations of salts present in sea water is considered to exert some bactericidal action (1). The effect of salts, however, seems insufficient to account alone for the short survival time of e. g. sewage bacteria in sea water.

Tests on different samples of water indicated that there existed some relation between the salinity and the virus inactivating capacity. There were no indications that oxidative inactivation of virus contributed significantly to a loss of viral infectivity and it was shown previously (5) that just heating the sea water (45° C or more for one hour) destroyed its virus inactivating capacity. It seemed therefore improbable that the salts of the water should be responsible for the low stability of suspended viruses. More likely the salinity of the water

seemed to be of importance for the action of the virus inactivating factors the latter being ineffective at concentrations of NaCl below 0 1 M

The photodynamic inactivation of enteroviruses has been studied i a by *Wallis & Melnick* (11) Although enteroviruses are usually resistant to photodynamic inactivation they are photosensitive under certain experimental conditions To exclude the possibility that a photodynamic action was involved in the inactivation of virus occurring in sea water inactivation tests with and without exposure of the water to light were performed No results indicating the presence of a photodynamic inactivation were obtained

In his review of the literature regarding survival of enteric microorganisms in sea water *Greenberg* (1) emphasizes that sea water contains a potent toxic factor which is thermolabile He concludes that the single most important factor in reducing the number of enteric bacteria in sea water is a biologic one and most likely is the result of the production of antibiotic substances by marine bacteria As yet it cannot be stated whether the same bactericidal factor causes the inactivation of viral infectivity Moreover the relationship between microorganisms living in sea water and the virus inactivation is unknown A study on the possible anti viral effect of such microorganisms has therefore been initiated and will be reported later It seems implausible that the reduction in demonstrable infective virus should be a result of adsorption or ingestion of virus by water microorganisms The characteristic course of the inactivation the heat lability and the finding that amino groups of proteins and amino acids are able to inhibit the antiviral activity (5) suggest reaction(s) of biochemical nature as responsible for the viral inactivation

SUMMARY

Sea water was found to possess a potent virus inactivating capacity This function of sea water required a concentration of NaCl of 0 1 M or higher but was not directly caused by the salinity of the water The inactivation of virus could not be explained in terms of oxidative inactivation or as a photodynamic reaction All of 8 different animal viruses were markedly unstable when suspended in sea water The characteristic course of the inactivation is described

REFERENCES

1 *Greenberg A F* Survival of enteric organisms in sea water Public Health Reports *71* 7 1956
2 *Kallings L O* Studies on the inactivation of bacterial viruses by normal human serum Acta path et microbiol scand nav *51* 389 1961
3 *Albertsson P A* Partition of cell particles and macromolecules Almqvist and Wiksell Uppsala 1960
4 *Karber G* Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche Arch exper Path *162* 480 1931

5 *Lycke E Magnusson Sigyn & Lund Ebba* Studies on the nature of the virus inactivating capacity of sea water Arch ges Virusforsch *17* 409 1965
6 *Lund Ebba & Lycke E* Effect of oxidation and reduction on the infectivity of poliomyelitis virus Arch ges Virusforsch *11* 100 1961
7 *Hedström C E & Lycke E* An experimental study on oysters as virus carriers Am J Hyg *79* 134 1964
8 *Plissier M & Therre P* Recherches sur l inactivation *in vitro* du poliovirus dans l eau de mer Ann Inst Pasteur *101* 840 1961
9 *Plissier M Therre P Stone E Broquet M & Broquet B* Recherches sur l inactivation *in vitro* du poliovirus dans l eau de mer II Experimentation sur dix mois Ann Inst Pasteur *103* 665 1962
10 *Plissier M* L inactivation dans l eau de mer et l eau d alimentation de certains enterovirus Arch ges Virusforsch *13* 76 1963
11 *Wallis C & Melnick J L* Photodynamic inactivation of enteroviruses J Bact *89* 41 1965

Acta path et microbiol scandinav 66 560 1966

BRIEF REPORT

A MICRORADIOGRAPHIC STUDY OF SALICYLATE-INDUCED SKELETAL ANOMALIES IN MOUSE EMBRYOS

By K S *Larsson* B *Ivemark* and B *Engfeldt*

Sodium salicylate has been shown to produce malformations in ribs and vertebrae in mouse embryos (2) The malformations consist of changes in the number of ribs and vertebrae and of pathological fusion of bone anlagen These types of malformation have been observed in infants too The teratogenic action of salicylates in mice has been proposed to be related to the depression of acid mucopolysaccharide synthesis (2) The aim of the present study was to look for possible disturbances in the mineralization process and for cellular abnormalities in embryos from salicylate treated mothers

Pregnant primiparous A/Jax mice mated overnight were used Vaginal plug could easily be observed in most cases the following morning and that day was denoted as the zero day of pregnancy (1) Sodium salicylate 10 mg/ 20 g body weight in 0 1 ml of distilled water was given i m in a single dose on the 9th gestation day The fetuses from 19 litters from salicylate treated mothers and 8 litters from untreated control animals were removed on the 18th gestation day The living fetuses from one uterine horn were fixed in 95 per cent ethanol for skeletal staining with Alizarin red S (3) Out of 70 investigated fetuses anomalies of ribs and vertebrae were found to occur in 70 respectively 61 per cent in the experimental group In the control group none of the 40 fetuses investigated showed these skeletal malformations

Living fetuses from the other horn were fixed in 10 per cent neutral formalin for light microscopy and microradiography (4) Transverse blocks (5 mm) from the thorax of the fetuses were embedded in paraffin without decalfication Sections (5 μ) were exposed at 8 12 A in an X-ray microscope designed by *Engstrom et al* (1957) Thus sections were examined from vertebrae and ribs from 37 fetuses The mineralization process was found to be normal for the age in all the fetuses No abnormalities on the tissue or cellular level could be found in the sections studied with the above mentioned technique

References 1 *Larsson K S* Acta Morphol Neerl Scand 4 349 1962 —2 *Larsson K S & Boström H* Acta Paediat Scand 54 43 1965 —3 *Dawson A B* Stain Technol *1* 123 1926 28 —4 *Engström A Lundberg B & Bergendahl G* J Ultrastructure Research *1* 147 1957

Received 2 ix 66 *from the Department of Pathology II University of Uppsala Department of Pediatric Pathology and Department of Anatomy and Histology Karolinska Institutet Stockholm Sweden*

This study has been supported by grants from the *Association for the Aid of Crippled Children* N Y U S A

INDEX

VOL 66 FASC 1–4 1966

SUPPLEMENTA